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TITLE OF THE INVENTION

METHODS FOR GENERATING MUTANT VIRUS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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____ The Commissioner is hereby authorized to charge the filing fee and any other charge for the present application to the account of the undersigned, Deposit Account No. 04-1406. A duplicate copy of this sheet is enclosed.	PROVISIONAL FILING FEE AMOUNT(S) $160.00

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Respectfully submitted

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Methods for Generating Mutant Virus

Field of the Invention

The present invention relates to a nucleic acid vector for delivery of a nucleic acid cassette to an insertion site in a selected viral genome, to methods of generating mutant virus using said vector and to the mutant HSV generated, and particularly, although not exclusively, to nucleic acid vectors for use in generating mutant herpes simplex virus.

Background to the Invention

Existing procedure for generating herpes simplex virus (HSV) mutants requires generation of a unique plasmid by cloning an entire expression cassette consisting of a promoter, gene of interest and polyadenylation sequences into a plasmid separately constructed to contain the relevant flanking sequences and then co-transfecting BHK cells with the resultant plasmid and HSV-1 DNA. Homologous recombination drives the formation of recombinant HSV-1 expressing the gene of interest, which is identified by Southern blotting. The recombinant virus is plaque purified 3-4 times by Southern blotting. This process takes several months.

This approach was taken by Liu et al[1] in generating two distinct plasmids, the first consisting of HSV-1 strain 17+ Sau3A fragment derived sequences flanking an expression cassette consisting of a CytoMegalovirus (CMV) promoter, Green Fluorescent Protein (GFP) gene and bGH polyadenylation (polyA) signal and the second wherein the

GFP gene is replaced with either a human or mouse Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) gene.

Shuttle vectors have been used to generate recombinant adenoviral vectors, e.g. the pAdEasy™ system of vectors (Stratagene), for use in overexpressing recombinant proteins in mammalian cells. However, these vectors require the cloning of the gene of interest into a first shuttle vector which is then contransformed into a specially constructed cell line to generate a recombinant adenoviral plasmid which is transfected into a separate specially constructed mammalian cell line in which the recombinant adenoviral plasmid is directly packaged into virus particles.

The HSV genome comprises two covalently linked segments, designated long (L) and short (S). Each segment contains a unique sequence flanked by a pair of inverted terminal repeat sequences. The long repeat (RL) and the short repeat (RS) are distinct.

The HSV ICP34.5 (also γ34.5) gene, which has been extensively studied[1,6,7,8], has been sequenced in HSV-1 strains F[9] and syn17+[3] and in HSV-2 strain HG52[4]. One copy of the ICP34.5 gene is located within each of the RL repeat regions. Mutants inactivating both copies of the ICP34.5 gene (i.e. null mutants), e.g. HSV-1 strain 1716[2] or the mutants R3616 or R4009 in strain F[5], are known to lack neurovirulence, i.e. be avirulent, and have utility as both gene delivery vectors or in the treatment of tumours by oncolysis. HSV strain 1716 has a 759bp

deletion in each copy of the ICP34.5 gene located within the BamHI s restriction fragment of each RL repeat.

ICP34.5 null mutants such as 1716 are, in effect, first-generation oncolytic viruses. Most tumours exhibit individual characteristics and the ability of a broad spectrum first generation oncolytic virus to replicate in or provide an effective treatment for all tumour types is not guaranteed.

Recombinant adenovirus and recombinant retrovirus[10] expressing nitroreductase have been constructed for use with the prodrug CB1954 with the intention of providing a treatment for cancer. The recombinant virus is not oncolytic and relies on gene directed enzyme-prodrug therapy to achieve tumour cell kill.

The prior art provides technically challenging, procedurally slow and inefficient materials and methods for generating recombinant HSV. In particular the prior art does not provide methods of, and materials for, generating recombinant HSV which are easy to detect, may be designed to be specific null mutants and which may express a selected gene of interest.

First generation oncolytic viruses such as HSV-1 strain 1716 show significant therapeutic potential in tumour and gene therapy. Overcoming the existing technical difficulties by enabling rapid generation and screening of second generation oncolytic viruses of this kind provides a significant improvement in the development of novel pharmaceutical compositions, vaccines and medicaments for the treatment of cancer and disease.

Summary of the Invention

The inventors have provided a generic plasmid vector designated RL1.dIRES-GFP. RL1.dIRES-GFP provides a platform for generating a plurality of 'shuttle vectors' which can exploit the process of homologous recombination to transfer a nucleotide sequence of interest (downstream of a selected promoter) into the disabling RL1 locus of HSV-1, generating easily identifiable, oncolytic, ICP34.5 null HSV-1 mutants expressing the products of the nucleotide sequence of interest, e.g. an RNA transcript or a polypeptide, and GFP. RL1.dIRES-GFP thus provides for ease of generation and purification of ICP34.5 null HSV.

RL1.dIRES-GFP is a useful vector for making second-generation oncolytic viruses having enhanced cytotoxic potential and which may express the product(s) of selected gene(s) to enhance the oncolytic and/or therapeutic effect of the administered virus.

The RL1.dIRES-GFP plasmid incorporates a multi-cloning sequence (MCS), upstream of an internal ribosome entry site (IRES), the GFP gene and SV40 polyadenylation sequences flanked by HSV-1 RL1 sequences. Incorporation of the encephalomyocarditis virus IRES (EMCV IRES) permits translation of two open reading frames from a single transcribed mRNA.

Following generation of a specific shuttle vector by cloning of the nucleotide sequence of interest (and the selected promoter) into RL1.dIRES-GFP, recombinant HSV-1

expressing the desired nucleic acid transcript or protein, can be generated and purified within 2 weeks. This compares with 2-3 months using prior art protocols.

In the ICP34.5 null HSV generated using the RL1.dIRES-GFP plasmid provided by the inventors transcription of both the nucleotide sequence of interest and GFP as a single transcript is controlled by the same promoter upstream of the nucleotide sequence of interest, the transcribed IRES directing cap-independent translation of GFP. The generated ICP34.5 null HSV are non-neurovirulent. By modifying the RL1.dIRES-GFP plasmid to incorporate appropriate flanking sequences surrounding the cassette other gene-specific HSV null mutants expressing GFP can be generated.

RL1.dIRES-GFP is promoterless, thus enabling a promoter of choice to be incorporated in the homologously recombined shuttle vector for controlling expression of the nucleotide sequence of interest from the inserted cassette.

Plasmid RL1.dIRES-GFP or modified plasmid shuttle vectors thereof further comprising nucleotide sequence encoding a nucleic acid transcript or polypeptide of interest may be provided in isolated or purified form.

By using the plasmid RL1.dIRES-GFP to generate a shuttle vector, designated RL1.dCMV-NTR-GFP, containing the E.coli nitroreductase gene downstream of a CMV IE promoter, both inserted at the MCS, the inventors have further provided a novel second generation oncolytic mutant HSV. The genome of this mutant HSV comprises the

heterologous E.coli nitroreductase protein coding sequence inserted at one or each ICP34.5 locus, disrupting the ICP34.5 protein coding sequence such that the ICP34.5 gene is non-functional and cannot express a functional ICP34.5 gene product. The generated HSV is capable of expressing the E.coli nitroreductase gene product under control of the inserted promoter. This virus thus has the oncolytic activity of HSV strain 1716 and can be used in gene directed enzyme-prodrug therapy and has shown significantly enhanced tumour cell killing in vitro when used with the prodrug CB1954. The mutant virus is designated HSV1716/CMV-NTR/GFP.

The inventors have also used plasmid RL1.dIRES-GFP to generate a shuttle vector, designated RL1.dCMV-asSCCRO-GFP, containing the human antisense squamous cell carcinoma related oncogene (SCCRO) arranged in an orientation downstream of a CMV IE promoter to produce antisense RNA transcripts for use in antisense therapeutic methods. Using this shuttle vector the inventors have provided another novel second generation mutant HSV, designated HSV1716/CMV-asSCCRO/GFP. The genome of this mutant HSV comprises the heterologous antisense SCCRO nucleotide sequence inserted at one or each ICP34.5 locus, disrupting the ICP34.5 protein coding sequence such that the ICP34.5 gene is non-functional and cannot express a functional ICP34.5 gene product. The generated HSV is capable of expressing an antisense RNA transcript under control of the CMV IE promoter which is capable of inhibiting the action of the SCCRO gene by binding to sense SCCRO nucleotide sequences, e.g. genomic SCCRO. This virus retains the oncolytic activity of HSV

strain 1716 and can be used in targeted antisense nucleotide delivery strategies and therapeutic methods.

At its most general the present invention comprises a nucleic acid vector for delivery of a nucleic acid cassette to an insertion site in a selected viral genome. The present invention further comprises novel HSV mutants which may be generated using the nucleic acid vector or vectors derived therefrom and methods for the generation of such vectors and HSV mutants.

According to a first aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking an insertion site in the genome of a selected herpes simplex virus strain; and
a cassette located between said first and second nucleotide sequences comprising nucleic acid encoding:

a) one or a plurality of insertion sites; and
b) a ribosome binding site; and
c) a marker.

According to a second aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking an insertion site formed in, or comprising all or a part of, the ICP34.5 protein coding sequence of the genome of a selected herpes simplex virus strain; and
a cassette located between said first and second nucleotide sequences comprising nucleic acid encoding:

a) one or a plurality of insertion sites; and

b) a ribosome binding site; and

c) a marker.

In the first and second aspects it is preferable for the cassette to comprise a plurality of insertion sites, each insertion site preferably formed by nucleic acid encoding a specific restriction endonuclease site ('restriction site'). Together the restriction sites may form a multiple cloning site (MCS) comprising a series of overlapping or distinct restriction sites, preferably a series of distinct restriction sites comprising one or more of the ClaI, BglII, NruI, XhoI restriction sites.

In the first and second aspects of the invention the encoded components of the cassette are preferably arranged in a predetermined order. In a preferred arrangement, the one or plurality of insertion sites is/are arranged upstream (i.e. 5') of the ribosome binding site and the ribosome binding site is arranged upstream (i.e. 5') of the marker.

According to a third aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking an insertion site in the genome of a selected herpes simplex virus strain; and
a nucleic acid cassette located between said first and second nucleotide sequences comprising:

a) a third nucleotide sequence being of interest; and nucleic acid encoding:

b) a ribosome binding site; and

c) a marker.

According to a fourth aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking an insertion site formed in, or comprising all or a part of, the ICP34.5 protein coding sequence of the genome of a selected herpes simplex virus strain; and
a nucleic acid cassette located between said first and second nucleotide sequences comprising:

a) a third nucleotide sequence being of interest; and nucleic acid encoding:
b) a ribosome binding site; and
c) a marker.

Vectors according to the third and fourth aspects may further comprise one or a plurality of insertion sites, more preferably restriction endonuclease sites, encoded by nucleic acid of the cassette.

According to a fifth aspect of the present invention there is provided a mutant herpes simplex virus wherein the herpes simplex virus genome comprises a nucleic acid cassette comprising, consisting or consisting essentially of:

a) a nucleotide sequence of interest; and nucleic acid encoding:
b) a ribosome binding site; and
c) a marker.

The mutant HSV is preferably a gene specific null mutant, more preferably an ICP34.5 null mutant. Preferably, the mutant HSV is generated by site directed insertion of the cassette into the viral genome, more preferably by homologous recombination.

The mutant HSV may be derived from a strain of either HSV-1 or HSV-2.

The use of such mutant HSV in the treatment of disease, including the treatment of tumours/cancer, preferably by oncolysis is provided. Use of such mutant HSV in the manufacture of a medicament for use in these treatments is also provided.

Mutant HSV of the fifth aspect are also provided for use in methods of medical treatment.

Medicaments comprising HSV mutants according to the fifth aspect for use in oncotherapy and methods of treating tumours comprising administering to a patient in need of treatment an effective amount of a mutant HSV according to the fifth aspect or a medicament comprising or derived from such HSV are also provided.

In the third, fourth and fifth aspects the nucleotide sequence of interest contained in the cassette preferably encodes a polypeptide of interest, or fragment thereof, or comprises selected antisense DNA, that is DNA corresponding to a gene component, e.g. regulatory sequence, 5' UTR, 3'UTR or protein coding sequence, or fragment of a gene component, which is inserted in the cassette in an orientation such that upon transcription

an antisense RNA is obtained. Thus the expressed product of the nucleotide sequence of interest may ultimately be a polypeptide, complete or truncated, or an antisense nucleic acid, preferably RNA.

By antisense nucleic acid is meant a nucleic acid having substantial sequence identity to the nucleic acid formed by the sequence of complementary bases to the single strand of a target nucleic acid. Thus, the antisense nucleic acid is useful in binding the target nucleic acid and may be used as an inhibitor to prevent or disrupt the normal activity, folding or binding of the target nucleic acid. The substantial sequence identity is preferably at least 50% sequence identity, more preferably at least 60, 70, 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, 99 or 100 identity. Identity of sequences is determined across the entire length of a given nucleotide sequence.

Where the nucleotide sequence of interest encodes a polypeptide of interest the polypeptide may be any selected polypeptide. Preferably, the polypeptide of interest is an heterologous or exogenous polypeptide (i.e. a non-HSV originating polypeptide), preferably a bacterial polypeptide, alternatively a mammalian polypeptide or a human polypeptide. The heterologous polypeptide may be useful in gene directed enzyme-prodrug targeting techniques for tissue specific delivery of active pharmaceutical agents. For example, the polypeptide of interest may be the Noradrenaline transporter (NAT), preferably bovine NAT, Sodium iodide symporter (NIS), Nitroreductase (NTR), preferably E.coli NTR, Endothelial nitric oxide synthase (eNOS),

Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) or a cytokine.

Where the nucleotide sequence of interest comprises an antisense nucleic acid, the antisense nucleic acid may comprise all or a fragment of the antisense squamous cell carcinoma related oncogene (SCCRO), preferably human SCCRO.

In the third, fourth and fifth aspects the cassette preferably further comprises a regulatory nucleotide sequence such as one or more selected promoter or enhancer elements known to the person skilled in the art, e.g. the CytoMegalovirus (CMV) promoter. The regulatory nucleotide sequence is preferably located upstream (i.e. 5') of the nucleotide sequence of interest and has a role in controlling and regulating transcription of the nucleotide sequence of interest and hence expression of the resulting transcript or polypeptide.

In the third, fourth and fifth aspects the components of the cassette are preferably arranged in a predetermined order. In a preferred arrangement, the nucleotide sequence of interest is arranged upstream (i.e. 5') of the ribosome binding site and the ribosome binding site is arranged upstream (i.e. 5') of the marker. Thus during transcription a single transcript may be produced from the cassette comprising a first cistron comprising the nucleotide sequence of interest and a second cistron encoding the marker wherein the ribosome binding site is located between the cistrons.

The following preferred arrangements are in appropriate accordance with any one or more of the first to fifth aspects described above.

A suitable ribosome binding site comprises a ribosome entry site permitting entry of a ribosome to the transcribed mRNA encoded by the nucleic acid of the cassette such that the ribosome binds to the translation start signal. Preferably, the ribosome entry site is an internal ribosome entry site (IRES), more preferably an encephalomyocarditis virus IRES, permitting cap-independent initiation of translation. The IRES thus enables translation of a coding sequence located internally of a bi- or poly- cistronic mRNA, i.e. of a cistron located downstream of an adjacent cistron on a single transcript.

Preferably the marker is a defined nucleotide sequence coding for a polypeptide which can be expressed in a cell line (e.g. BHK cells) infected with mutant herpes simplex virus into which the cassette has been recombined. The function of the marker is to enable identification of virus plaques containing mutant virus transformed with the cassette.

Alternatively, the marker may comprise a defined nucleotide sequence which can be detected by hybridisation under high stringency conditions with a corresponding labelled nucleic acid probe, e.g. using a fluorescent- or radio-label.

The marker is preferably a detectable marker, more preferably an expressible marker polypeptide or protein

comprising at least the coding sequence for the selected polypeptide or protein. The nucleic acid encoding the marker may further comprise regulatory sequence upstream and/or downstream of the coding sequence having a role in control of transcription of the marker mRNA. Preferred markers include the Green Fluorescent Protein (GFP) protein coding sequence or gene, preferably the enhanced Green Fluorescent Protein (EGFP) protein coding sequence or gene.

In another preferred arrangement, the cassette further comprises a polyadenylation sequence ('polyA sequence'). Preferably the polyA sequence comprises the Simian Virus 40 (SV40) polyA sequence. The preferred location of the polyA sequence within the cassette is immediately downstream (i.e. 3') of the marker.

The first and second nucleotide sequences preferably comprise nucleotide sequences having identity to regions of the genome surrounding the insertion site in the selected herpes simplex virus strain (the 'viral insertion site'). These sequences enable the cassette to be incorporated at the viral insertion site by homologous recombination between the first and second nucleotide sequences and their respective corresponding sequences in the viral genome.

Thus the first and second nucleotide sequences are flanking sequences for homologous recombination with corresponding sequences of a selected viral genome, such homologous recombination resulting in insertion of the cassette at the viral insertion site.

Preferably, the first and second nucleotide sequences correspond to nucleotide sequences flanking an insertion site in the RL1 locus of the HSV genome, more preferably in the ICP34.5 protein coding sequence of the HSV genome.

Preferably, the first and second nucleotide sequences are each at least 50bp in length, more preferably at least 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000bp in length. Preferably, each of the first and second nucleotide sequences have at least 50% sequence identity to their corresponding sequence in the viral genome, more preferably at least 60%, 70%, 75%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% 99% or 100% identity. Identity of sequences is determined across the entire length of a given nucleotide sequence.

The first and second nucleotide sequences may be characterised by the ability of one strand of a given sequence to hybridise with the corresponding single-stranded complement of the HSV genome under varying hybridisation stringency conditions. Suitably, the first and second nucleotide sequences will hybridise with their corresponding complement under very low, low or intermediate stringency conditions, more preferably at high or very high stringency conditions.

The nucleotide sequence of interest which forms part of the inserted cassette may encode a full length transcript or polypeptide (i.e. comprise the complete protein coding sequence). Alternatively, the nucleotide sequence of

interest may comprise one or more fragments of the full length sequence respectively coding for a fragment of the full length transcript or a truncated polypeptide or antigenic peptide respectively. A fragment may comprise a nucleotide sequence encoding at least 10% of the corresponding full length sequence, more preferably the fragment comprises at least 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 97, 98 or 99% of the corresponding full length sequence. Preferably, the fragment comprises at least 30 nucleotides, more preferably at least 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000 nucleotides.

The viral insertion site is the position between the genomic nucleotide sequences corresponding to the first and second nucleotide sequences of the vector (the 'genomic' and 'vector flanking sequences' respectively) at which homologous recombination will occur and may be predetermined by selection of the vector flanking sequences. Where the genomic flanking sequences are immediately adjacent, the insertion site is the position between the peripheral and immediately adjacent bases of the two genomic flanking sequences, such that insertion of the cassette separates the genomic flanking sequences. Where the genomic flanking sequences are separated by one or a plurality of bases in the viral genome, the insertion site is formed by said one or a plurality of bases which are excised from the genome by the homologous recombination event.

The position of the viral insertion site may be accurately selected by careful selection and construction of the vector flanking sequences. Accordingly, the vector may be constructed such that homologous insertion of the cassette results in disruption of a chosen protein coding sequence and inactivation of the respective gene product or such that the cassette is inserted at a non-protein coding region of the viral genome. The complete genome sequences of several herpes simplex virus strains have been reported and are publicly available. The complete genome sequence for HSV-1 strain 17syn+ was reported by Dolan et al[3] (incorporated herein by reference) and the complete genome sequence of HSV-2 strain HG52 was reported by Dolan et al[4] (incorporated herein by reference) and is available from the EMBL database under accession code Z86099. Using this information, the vector of the present invention may preferably be designed for use in generating mutant HSV-1 (e.g. in strain 17 or F) or mutant HSV-2 (e.g. in strain HG52).

Preferably the first and second nucleotide sequences (vector flanking sequences) each comprise sequence corresponding to the RL terminal repeat region of the genome of the selected HSV (e.g. HSV-1 strains 17 or F or HSV-2 strain HG52). More preferably, vector flanking sequences comprise, consist or consist essentially of nucleotide sequences of the RL repeat region which flank the ICP34.5 protein coding sequence. In flanking the ICP34.5 coding sequence, one or both of the selected sequences may, in the corresponding HSV genome, overlap, i.e. extend into, the ICP34.5 protein coding sequence or one or both sequences may be selected so as to not overlap the ICP34.5 protein coding sequence. In a similar

manner, the selected sequences may be chosen to overlap completely or partially other important encoded signals, e.g. transcription initiation site, polyadenylation site, defined promoters or enhancers. In this preferred arrangement the insertion site will thus comprise all or a part of the ICP34.5 protein coding sequence and/or be such that the inserted cassette disrupts the ICP34.5 protein coding sequence.

Thus, vectors according to the present invention comprising first and second nucleotide sequences corresponding to regions of the RL repeat region flanking and/or overlapping the ICP34.5 protein coding sequence may be used in the generation of ICP34.5 null mutants wherein all or a portion of the ICP34.5 protein coding sequence is excised and replaced during the homologous recombination event such that both copies of the ICP34.5 coding sequence are disrupted. Successfully transformed virus are thus mutants incapable of generating the ICP34.5 active gene product.

Preferably, each component of the cassette is positioned substantially adjacent the neighbouring component such that a single bicistronic transcript comprising or consisting essentially of the mRNA encoding the nucleotide sequence of interest, ribosome binding site and marker is obtainable.

Preferably, the vector further comprises, consists, or consists essentially of a nucleic acid encoding a selectable marker such as a polypeptide or protein conferring antibiotic resistance e.g. kanamycin resistance or ampicillin resistance.

A vector of the present invention preferably comprises a DNA vector, particularly a dsDNA vector. The vector may be provided as a linear or circular (plasmid) DNA vector. The vector preferably contains nucleotide sequences, e.g. restriction endonuclease site(s), permitting transition between the two forms by use of DNA ligation and restriction materials (e.g. enzymes) and techniques known to the person skilled in the art. To achieve homologous recombination with a selected HSV strain, the vector is preferably provided in linear form.

In one preferred arrangement, the vector is plasmid RL1.dIRES-GFP deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

In another preferred arrangement, the vector is a variant of plasmid RL1.dIRES-GFP.

Vectors according to the present invention are preferably constructed for use in generating engineered HSV-1 or HSV-2 by insertion of a nucleic acid cassette through a mechanism of homologous recombination between nucleotide

sequences flanking the cassette and corresponding sequences in the selected herpes simplex virus genome.

Thus, vectors according to the present invention may comprise and have use as:

i) gene delivery (gene therapy) vectors for delivery of a selected protein coding sequence or antisense nucleic acid to a specific locus of the HSV genome; and/or

ii) expression vectors for expression of the delivered protein coding sequence or antisense nucleic acid of i) from the HSV genome under the control of a selected regulatory element; and/or

iii) vectors for the generation of HSV gene-specific null mutants wherein the cassette is inserted at a selected genomic location to disrupt the protein coding sequence of a selected HSV gene such that the gene product is inactive in the resultant mutant virus.

Vectors according to the present invention may be used in the manufacture of engineered gene specific HSV null mutants, i.e. HSV mutants incapable of expressing an active gene product of a selected gene. Such vectors may also be used in the manufacture of a medicament, preferably comprising said gene specific HSV null mutant, for use in treating tumours, preferably by the oncolytic treatment of the tumour. Preferably, such tumours may be primary or secondary (metastatic) tumours originating either in the central or peripheral nervous system, e.g. glioma, medulloblastoma, meningioma, neurofibroma, ependymoma, Schwannoma, neurofibrosarcoma, astrocytoma and oligodendroglioma, or originating in non-nervous

system tissue e.g. melanoma, mesothelioma, lymphoma, hepatoma, epidermoid carcinoma, prostate carcinoma, breast cancer cells, lung cancer cells or colon cancer cells. HSV mutants generated using vectors of the present invention may be used to treat metastatic tumours of the central or peripheral nervous system which originated in a non-nervous system tissue. In one embodiment of the present invention, there is provided an attenuated replication competent HSV expressing antisense SCCRO, namely, HSV1716asSCCRO which may be used in the treatment of squamous cell cancer, particularly head and neck squamous cell cancer.

Accordingly the invention further provides a pharmaceutical composition comprising HSV 1716asSCCRO.

Vectors according to the present invention may also be used in the manufacture of engineered HSV mutants wherein the genome of the mutant HSV comprises an exogenous gene which has been inserted in the HSV genome by homologous recombination of the cassette. Preferably, the exogenous gene is expressed in the mutant HSV, which expression may be regulated by a regulatory element, e.g. promoter, forming part of the inserted cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

Vectors according to the present invention may also be used in the manufacture of an engineered HSV mutant wherein the genome of the mutant HSV comprises an exogenous gene (i.e. a non-HSV originating gene) which

has been inserted in a protein coding sequence of the HSV genome by homologous recombination of the cassette such that the mutant HSV is incapable of expressing the active gene encoded by said protein coding sequence and wherein the exogenous gene product is expressed under the control of a regulatory element. Preferably, the regulatory element forms part of the cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

Vectors according to the present invention may also be used in the manufacture of engineered HSV mutants wherein the genome of the mutant HSV comprises a nucleotide sequence which has been inserted in the HSV genome by homologous recombination of the cassette such that the nucleotide sequence is arranged to be transcribed from the HSV genome under the control of a regulatory element e.g. promoter, preferably a regulatory element forming part of the inserted cassette, to produce an antisense transcript. Preferably the antisense nucleotide sequence is an exogenous (i.e. non-HSV originating) sequence. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

Vectors according to the present invention may also be used in the manufacture of an engineered HSV mutant wherein the genome of the mutant HSV comprises a nucleotide sequence which has been inserted in a protein coding sequence of the HSV genome by homologous

recombination of the cassette such that the mutant HSV is incapable of expressing the active gene encoded by said protein coding sequence and wherein the inserted nucleotide sequence is expressed under the control of a regulatory element to produce an antisense transcript. Preferably, the regulatory element forms part of the cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

In a sixth aspect of the present invention there is provided a method of generating a nucleic acid vector comprising the steps of:

i) providing a first nucleotide sequence comprising a predetermined second nucleotide sequence corresponding to a selected nucleotide sequence of the genome of an HSV strain; and

ii) inserting nucleotide sequences in said second nucleotide sequence encoding:

   a) one or a plurality of insertion sites and/or a nucleotide sequence of interest; and

   b) a ribosome binding site; and

   c) a marker.

Preferably, in the sixth aspect described above, the inserted nucleotide sequences separates the second nucleotide sequence into two vector flanking sequences, the inserted nucleotide sequences forming a cassette therebetween.

According to a seventh aspect of the present invention there is provided a method of generating mutant HSV

comprising inserting a cassette comprising nucleotide sequences encoding:

a) one or a plurality of insertion sites and/or a nucleotide sequence of interest; and

b) a ribosome binding site; and

d) a marker

into the genome of a selected HSV strain, said method comprising the steps of:

i) providing a vector according to any of the first to fourth or sixth aspects;

ii) where the vector is a plasmid, linearising the vector; and

iii) co-transfecting a cell culture with the linearised vector and genomic DNA from said HSV strain.

Preferably, said co-transfection is carried out under conditions effective for homologous recombination of said cassette into an insertion site of the viral genome.

Preferably, said method further comprises one or more of the steps of:

1) screening said co-transfected cell culture to detect mutant HSV expressing said marker; and/or

2) isolating said mutant HSV; and/or

3) screening said mutant HSV for expression of the nucleotide sequence of interest or the RNA or polypeptide thereby encoded; and/or

4) screening said mutant HSV for lack of an active gene product; and/or

5) testing the oncolytic ability of said mutant HSV to kill tumour cells in vitro.

In an eighth aspect of the present invention there is provided a method of lysing or killing tumour cells in vitro or in vivo comprising the step of administering mutant HSV, having a mutation in each ICP34.5 protein coding sequence and generated by a method according to the seventh aspect, to tumour cells.

In an ninth aspect of the present invention there is provided a method of treating a tumour comprising administering to a subject mutant HSV having a mutation in each ICP34.5 protein coding sequence and generated by a method according to the eighth aspect.

In a tenth aspect of the present invention there is provided a medicament, pharmaceutical composition or vaccine comprising a mutant HSV generated by a method according to the seventh aspect. The medicament, pharmaceutical composition or vaccine is preferably for use in the oncolytic treatment of tumours and may further comprise a pharmaceutically acceptable carrier, adjuvant or diluent.

In an eleventh aspect of the present invention there is provided a kit of parts comprising a first container having a quantity of a vector according to any of the first to fourth aspects of the present invention and a second container comprising a quantity of HSV genomic DNA.

In a twelfth aspect of the present invention there is provided a mutant HSV generated using the vector of, or vectors derived from, the first to fourth aspects. Preferably, the mutant is a gene specific null mutant,

more preferably an HSV ICP34.5 null mutant, wherein the HSV genome comprises an inserted nucleotide sequence of interest encoding a selected antisense RNA or an heterologous polypeptide. Preferably the nucleotide sequence of interest has been inserted in each RL region of the HSV genome, more preferably at both of the ICP34.5 loci, still more preferably the inserted heterologous nucleic acid disrupts the ICP34.5 protein coding sequence such that both ICP34.5 genes are non-functional and the mutant HSV is incapable of expressing an active ICP34.5 gene product from the disrupted ICP34.5 protein coding sequences. Preferably, the mutant HSV is generated according to the method of the seventh aspect. Preferably, the inserted heterologous nucleotide sequence is non-endogenous to HSV and encodes a polypeptide of interest selected from the group comprising or consisting of Noradrenaline transporter (NAT), preferably bovine NAT, Sodium iodide symporter (NIS), Nitroreductase (NTR), preferably E.coli NTR, Endothelial nitric oxide synthase (eNOS), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) or a cytokine. Alternatively the inserted nucleotide sequence of interest encodes the antisense transcript of the squamous cell carcinoma related oncogene (SCCRO), preferably human SCCRO.

The inserted nucleotide sequence of interest is preferably expressed or capable of expression under the control of an inserted regulatory element, preferably the CMV IE promoter. The mutant HSV genome preferably encodes the GFP gene product. More preferably the GFP coding sequence and nucleotide sequence of interest are arranged to be transcribed on a single bicistronic transcript such that expression of GFP is an indicator of HSV gene

specific null mutants transfornmed with the nucleotide sequence of interest.

In one preferred arrangement, the mutant HSV is HSV1716/CMV-NTR/GFP deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 05 November 2003 at the European Collection of Cell Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03110501 in accordance with the provisions of the Budapest Treaty.

Use of mutant HSV according to the twelfth aspect in the preparation of a medicament for use in the treatment of disease such as the oncolytic treatment of tumours, comprising primary and/or secondary nervous system and/or non-nervous system tumours, and/or the treatment of disease by gene directed enzyme-prodrug therapy and/or the treatment of disease, including tumours, by the use of antisense RNA technology is provided. Compositions comprising mutant HSV of the twelfth aspect for use in treating such disease are also provided. Mutant HSV of the twelfth aspect are also provided for use in methods of medical treatment.

Medicaments comprising HSV mutants according to the twelfth aspect for use in oncotherapy and methods of treating tumours comprising administering to a patient in need of treatment an effective amount of a mutant HSV according to the twelfth aspect or a medicament comprising or derived from such HSV are also provided.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Sequence identity

In accordance with the present invention, the appropriate level of sequence identity between the first and second nucleotide sequences of the vector and the corresponding nucleotide sequences of the HSV genome may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., ("Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence identity

is to calculate the melting temperature T_m (Sambrook et al., 1989):

T_m = *81.5°C + 16.6Log [Na+] + 0.41(% G+C) - 0.63 (% formamide) - 600/n*

where n is the number of bases in the oligonucleotide.

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in identity. Thus, targets with greater than about 75% sequence identity across their entire length would be observed using a hybridization temperature of 42°C.

Accordingly, nucleotide sequences can be categorised by an ability to hybridise under different hybridisation and washing stringency conditions which can be selected by using the above equation.

Sequences exhibiting 95-100% sequence identity are considered to hybridise under very high stringency conditions, sequences exhibiting 85-95% identity are considered to hybridise under high stringency conditions, sequences exhibiting 70-85% identity are considered to hybridise under intermediate stringency conditions, sequences exhibiting 60-70% identity are considered to hybridise under low stringency conditions and sequences exhibiting 50-60%% identity are considered to hybridise under very low stringency conditions.

Brief Description of the Figures

Figure 1. Generation of plasmid RL1.dIRES-GFP from plasmids pNAT-IRES-GFP and RL1.del.

Figure 2. Agarose gel electrophoresis of *Hpa*I digested, CIP treated, RL1.del. RL1.del was digested with *Hpa*I. The digested DNA was then treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. *Hpa*I linearises the vector at 8.6 Kbp.

Figure 3. Agarose gel electrophoresis of *Nsi*I/*Ssp*I digested pNAT-IRES-GFP (A) and purified/blunt-ended pCMV-NAT-IRES-GFP-PolyA (B). Four *Nsi*I/*Ssp*I digestions of pNAT-IRES-GFP were electrophoresed, beside a 1Kbp DNA Ladder (Promega) on a 1% agarose gel. The 5.4Kbp fragments (pCMV-NAT-IRES-GFP-PolyA) were purified from the gel. The purified DNA was blunt ended using Klenow polymerase and a sample electrophoresed on an agarose gel to check its concentration.

Figure 4. Identification of RL1.del clones containing the pCMV-NAT-IRES-GFP-PolyA insert. Ligation reactions were set up with the purified, blunt ended pCMV-NAT-IRES-GFP-PolyA fragment and *Hpa*I digested, CIP treated RL1.del. Bacteria were transformed with samples from the ligation reactions and plated out onto LBA (Amp^r) plates. Colonies were picked and plasmid DNA was extracted and digested with *Afl*II. Digested samples were electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel.

*Clones 5 and 8 contained the pCMV-NAT-IRES-GFP-PolyA insert as two fragments of the predicted size - 4.8Kbp and 9.2Kbp - were generated from *Afl*II digestion. Clones without inserts would not be digested with *Afl*II as there is no *Afl*II site in RL1.del.

N.B. Inserts could have been cloned in two orientations, both of which were acceptable.

Figure 5. Determination of the orientation of pCMV-NAT-IRES-GFP-PolyA in clone 5 (RL1.dCMV-NAT-GFPb). pCMV-NAT-IRES-GFP-PolyA (blunt ended) could have been cloned into the *Hpa*I site of RL1.del in two orientations. To determine the orientation of the insert in clone 5, the plasmid was digested with *Xho*I and the digested DNA electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. If the insert had been cloned in the orientation shown in A, two fragments of 10.2Kbp and 3.8Kbp would be generated from *Xho*I digestion. If it had been cloned in the opposite orientation (B), two fragments of 12.4Kbp and 1.6Kbp would be generated. The presence of two fragments of 10.2Kbp and 3.8Kbp in the gel confirmed that the insert had been cloned in the orientation shown in A.

*This *Xho*I site was present in the initial cloning vector (RL1.del), upstream of the *Hpa*I site into which pCMV-NAT-IRES-GFP-PolyA was cloned.

Figure 6. Removal of pCMV-NAT from clone 5 (A) and large scale plasmid preparation of RL1.dIRES-GFP (B). Four samples of clone 5 were digested with *Xho*I and electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel (A). The larger fragment of DNA generated from this digestion (10.2Kbp) was purified from

the gel and ligated back together, at the *Xho*I sites, to form a single *Xho*I site in a new plasmid, designated RL1.dIRES-GFP. A large-scale plasmid preparation was grown up and the preparation checked by digesting with *Xho*I. 1μl and 4μl of the digested DNA was electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel (B). The DNA should produce a single fragment of 10.2Kbp when digested with *Xho*I. The *Cla*I, *Bgl*II, *Nru*I and *Xho*I sites of RL1.dIRES-GFP are all unique.
*Clone 5 is the RL1.del plasmid into which has been cloned the 5.4Kbp pCMV-NAT-IRES-GFP-PolyA fragment from pNAT-IRES-GFP.

Figure 7. Generation, detection and purification of ICP34.5 null HSV-1 expresssing a gene product of interest.

Figure 8. Strategy used to clone pCMV-NTR from pPS949 into RL1.dIRES-GFP. (1) Digest pPS949 with *Bam*H1 and purify the 1.6Kbp pCMV-NTR fragment; (2) Digest RL1.dIRES-GFP with *Bgl*II and treat with Calf Intestinal Phosphatase (CIP); (3) Clone the pCMV-NTR fragment (*Bam*HI ends) into the *Bgl*II site of RL1.dIRES-GFP.
* The pPS949 plasmid was a kind gift from Professor Lawrence Young (University of Birmingham) and contains the *E.coli* nitroreductase (NTR) gene downstream of the CMV-IE promoter (pCMV) in pLNCX (Clontech).

Figure 9. Agarose gel electrophoresis of *Bam*HI digested pPS949 (A) and the purified pCMV-NTR fragment (B). Four samples of pPS949 were digested with *Bam*HI and electrophoresed, beside a 1Kbp DNA ladder (L) (New

England Biolabs), on a 1% agarose gel. The 1.6Kbp fragments, consisting of the *E.coli* nitroreductase (NTR) gene downstream of the CMV IE promoter (pCMV), were purified from the gel and a sample of the purified DNA was electrophoresed on an agarose gel to check its concentration.

Figure 10. Agarose gel electrophoresis of *Bgl*II digested, CIP treated RL1.dIRES-GFP.RL1.dIRES.GFP was digested with *Bgl*II. The digested plasmid was then treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel to check its concentration. pCMV-NTR from pPS949 was subsequently cloned into this digested/CIP treated vector.

Figure 11. Determination of the orientation of pCMV-NTR in clone 4. pCMV-NTR (*Bam*HI ends) could have been cloned into the *Bgl*II site of RL1.dIRES-GFP in two orientations. To determine the orientation, clone 4 was digested with *Bgl*II and *Xho*I and the digested DNA electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. If the insert was in the desired orientation (A), two fragments (11.5Kbp and 300bp) would be generated. If in the opposite orientation, two fragments of 10.5Kbp and 1.3Kbp would be generated. The presence of a band at ~300bp (and the absence of a band at 1.3Kbp) confirmed that the pCMV-NTR fragment had been cloned into the vector in the desired orientation.

Figure 12. Agarose gel electrophoresis of *Sca*I digested clone 4 (A) and HSV1716/CMV-NTR/GFP viral titres (B). Clone 4 (RL1.dCMV-NTR-GFP) was digested with *Sca*I, the digested DNA purified and 5µl electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel, to check its concentration. 80% confluent BHK cells were then co-transfected with 10µl HSV17$^+$ DNA and an appropriate volume of the remaining digested clone 4. The cells were incubated at 37°C for 3 days until cpe was evident. Recombinant viral plaques were picked under the fluorescent microscope, purified and a virus stock, named HSV1716/CMV-NTR/GFP, grown up. The cell-associated and cell-released fraction of the virus stock was titrated on BHK cells.

Figure 13. Growth kinetics of HSV17$^+$, HSV1716 and HSV1716/CMV-NTR/GFP in confluent BHK and 3T6 cells. Confluent BHK and 3T6 cells were infected at a MOI of 0.1pfu/cell. Infected cells were harvested at 0, 4, 24, 48 and 72hrs post infection, sonicated and progeny virus titrated on BHK cell monolayers. All viruses replicated with similar kinetics in BHK cells (A); HSV1716 and HSV1716/CMV-NTR/GFP both failed to replicate efficiently in confluent 3T6 cells (B).

Figure 14. Western blot analysis of ICP34.5 expression in HSV17$^+$ and HSV1716/CMV-NTR/GFP infected BHK cells. BHK cells were infected with HSV17$^+$ and HSV1716/CMV-NTR/GFP at a MOI of 10pfu/cell. 16hrs post infection, the cells were harvested and protein extracts analysed using 10% SDS-PAGE in a Western blot using a polyclonal anti-ICP34.5 antibody. ICP34.5 was strongly expressed in HSV17$^+$

infected cells but was not expressed in HSV1716/CMV-NTR/GFP infected cells.

Figure 15. Western blot analysis of NTR expression in HSV1716/CMV-NTR/GFP infected cell lines. BHK, C8161, VM and 3T6 cells were infected with 10pfu/cell HSV1716/CMV-NTR/GFP, HSV17^{+} or mock infected. 16hrs post infection, the cells were harvested and protein extracts analysed in a Western blot using a polyclonal NTR-specific antibody. Significant NTR expression was detected in all the HSV1716/CMV-NTR/GFP infected cells. No NTR expression was detected in the mock or HSV17^{+} infected cells.

Figure 16. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP with or without CB1954 (50μM) on confluent 3T6 cells. Confluent 3T6 cells in three wells of a 96-well plate were mock infected, infected with 1 or 10pfu/cell HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of HSV1716-GFP. 45 minutes later, infected cells were overlaid with media containing 50μM CB1954 or with media alone and incubated at 37°C. 24, 48, 72, 96, and 120hrs later, % cell survival was determined relative to that of mock infected cells without prodrug using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Figures shown represent the mean of 3 values +/- standard error of the mean.

Figure 17. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP with or without CB1954 (50μM) on confluent C8161 cells. Confluent C8161 cells in three wells of a 96-well plate were mock infected, infected with 1 or 10pfu/cell HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of

HSV1716-GFP. 45 minutes later, infected cells were overlaid with media containing 50μM CB1954 or with media alone and incubated at 37°C. 24, 48 and 72hrs later, % cell survival was determined relative to that of mock infected cells without prodrug using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Figures shown represent the mean of 3 values +/- standard error of the mean.

Figure 18. Confluent 3T6 cells 72hrs post treatment with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell HSV1716/CMV-NTR/GFP with 50μM CB1954 (B). The extent of cell death is significantly more pronounced in HSV1716/CMV-NTR/GFP infected cells overlaid with media containing 50μM CB1954 than in HSV1716/CMV-NTR/GFP infected cells overlaid with normal media. The extent of cell death following infection of these cells with 10pfu/cell HSV1716, with or without CB1954, is comparable to that seen in A (data not shown). 50μM CB1954 alone has no effect on these cells.

Figure 19. Confluent C8161 cells 72hrs post treatment with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell HSV1716/CMV-NTR/GFP with 50μM CB1954 (B). The extent of cell death is significantly more pronounced in HSV1716/CMV-NTR/GFP infected cells overlaid with media containing 50μM CB1954 than in HSV1716/CMV-NTR/GFP infected cells overlaid with normal media. The extent of cell death following infection of these cells with 10pfu/cell HSV1716, with or without CB1954, is comparable to that seen in A (data not shown). 50μM CB1954 alone has no effect on these cells.

Figure 20. Strategy used to clone pCMV-asSCCRO, from pUSEamp-asSCCRO, into RL1.dIRES-GFP. (1) Digest pUSEamp-asSCCRO with *Ssp*1 and *Xho*I and purify the 1.96Kbp pCMV-asSCCRO fragment; (2) Digest RL1.dIRES-GFP with *Bgl*II, blunt end using Klenow polymerase and treat with Calf Intestinal Phosphatase (CIP). (3) Clone the blunt ended pCMV-asSCCRO fragment (1.96Kbp) into *Bgl*II digested/blunt ended/CIP treated RL1.dIRES-GFP. (*pUSEamp-asSCCRO was provided by Memorial Sloan-Kettering Cancer Centre, New York.)

Figure 21. Agarose gel electrophoresis of *Bgl*II digested, blunt ended, CIP treated RL1.dIRES-GFP. RL1.dIRES.GFP was digested with *Bgl*II. The digested plasmid was then blunt ended using Klenow polymerase and treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/blunt ended/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel to check its concentration. pCMV-asSCCRO was subsequently cloned into this digested/CIP treated vector.

Figure 22. Agarose gel electrophoresis of *Ssp*I/*Xho*I digested pUSEamp-asSCCRO (A) and the purified pCMV-asSCCRO fragment (B). Four samples of pUSEamp-asSCCRO were digested with *Ssp*I and *Xho*I then electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel. The 1.96Kbp fragments, consisting of DNA antisense to the squamous cell carcinoma related oncogene (asSCCRO) downstream of the CMV IE promoter (pCMV), were purified from the gel, blunt ended using Klenow polymerase,

purified again and a sample of the purified DNA electrophoresed on an agarose gel to check its concentration.

Figure 23. Identification of RL1.dIRES-GFP clones containing the pCMV-asSCCRO insert. Ligation reactions were set up with the purified, blunt ended pCMV-asSCCRO fragment and *Bgl*II digested, blunt ended, CIP treated RL1.dIRES-GFP. Bacteria were transformed with samples from the ligation reactions and plated onto LBA (Amp^r) plates. Colonies were picked and plasmid DNA was extracted and digested with *Bgl*II. Digested samples were electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel.
*Clone 11 contained the pCMV-asSCCRO insert as two fragments of the predicted size - 1.4Kbp and 10.8Kbp were generated from *Bgl*II digestion. Clones without the insert would not produce a fragment of 1.4Kbp when digested with *Bgl*II.

Figure 24. Determination of the orientation of pCMV-asSCCRO in clone 11. The presence of an *Nru*I site, ~320bp into the cloned pCMV-asSCCRO fragment, was utilized to determine the orientation of pCMV-asSCCRO. Clone 11 was digested with *Nru*I and electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel. If pCMV-asSCCRO was in the desired orientation (A), *Nru*I digestion would produce a fragment of 1.64Kbp. If in the opposite orientation (B), no 1.64Kbp fragment would be generated from this digestion. The presence of a fragment at 1.64Kbp in the gel confirmed that pCMV-asSCCRO was in the desired orientation. (*This *Nru*I site was already

present in the initial cloning vector (i.e. RL1.dIRES-GFP)).

Figure 25. Agarose gel electrophoresis of *Sca*I digested clone 11 (A) and HSV1716/CMV-asSCCRO/GFP virus titre (B). Clone 11 (RL1.dCMV-asSCCRO-GFP) was digested with *Sca*I, the digested DNA purified and 5µl electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel, to check its concentration. 80% confluent BHK cells were then co-transfected with 10µl HSV17$^+$ DNA and an appropriate volume of the remaining digested clone 11. The cells were incubated at 37°C for 3 days until cpe was evident. Recombinant viral plaques were picked under the fluorescent microscope, purified and a virus stock, named HSV1716/CMV-asSCCRO/GFP, grown up. HSV1716/CMV-asSCCRO/GFP was titrated on BHK cells.

Figure 26. Cytotoxicity assay for cell lines SCC15 and 584 after infection with HSV1716 or HSV1716asSCCRO at MOI of 1pfu/cell and 5pfu/cell.

Figure 27. Cytotoxicity assay for cell lines 1483 and 1986 after infection with HSV1716 or HSV1716asSCCRO at MOI of 1 pfu/cell and 5pfu/cell.

Figure 28. Cytotoxicity assay for cell line 1186 and 1386 after infection with HSV1716 or HSV1716asSCCRO at MOI of 1 pfu/cell and 5pfu/cell.

Figure 29. Viral proliferation assays for head and neck squamous cell carcinoma cell lines after infection with HSV1716 or HSV1716asSCCRO at MOI 1pfu/cell.

Figure 30. Infectivity assay- gfp expression 6 hours post infection with 1716gfp virus.

Figure 31. Western blot results of the cell line SCC15 showing downregulation of SCCRO protein at 12 hours with HSV1716asSCCRO but not in 584.

Figure 32. Nude mice xenograft growth curves in SCC15 and 584 following single intratumoural injection of HSV1716 or HSV1716asSCCRO.

Figure 33. Nude mice xenograft growth curves in SCC15 following single intratumoural injection of PBS, HSV1716 or HSV1716asSCCRO.

Detailed Description of the Best Mode of the Invention

Specific details of the best mode contemplated by the inventors for carrying out the invention are set forth below, by way of example. It will be apparent to one skilled in the art that the present invention may be practiced without limitation to these specific details.

Example 1

Construction of plasmid RL1.dIRES-GFP

General Approach

Plasmid RL1.dIRES-GFP was generated in three stages, illustrated in Figure 1.

1. The DNA sequences containing the CMV IE promoter (pCMV), the NAT gene, the internal ribosome entry site (IRES), the GFP reporter gene and the SV40 polyadenylation sequences were excised from pNAT-IRES-GFP using *Nsi*I and *Ssp*I and purified.

2. The purified pCMV-NAT-IRES-GFP-PolyA DNA fragment was cloned into RL1.del to form a new plasmid designated RL1.dCMV-NAT-GFP.

3. The pCMV-NAT DNA sequences of RL1.dCMV-NAT-GFP were excised using *Xho*I and the remainder of the plasmid re-ligated to form a novel plasmid designated RL1.dIRES-GFP. This novel plasmid contained a multi-cloning site (all sites shown are unique) upstream of an IRES, the GFP gene and the SV40 polyA sequences all within the HSV-1 RL1 flanking sequences. Recombinant ICP34.5 null HSV-1, expressing a gene of interest in the RL1 locus, can be generated by cloning the gene of interest (downstream of a suitable promoter) into the multi-cloning site and co-transfecting BHK cells with the plasmid and HSV-1 DNA. Recombinant virus expressing the target gene can be identified using GFP fluorescence.

Removal of the CMV promoter and noradrenaline transporter gene (pCMV-NAT) from RL1.dCMV-NAT-GFP, followed by re-ligation of the remainder of the plasmid, resulted in a novel plasmid (RL1.dIRES-GFP) containing a multi-cloning site (MCS), upstream of the encephalomyocarditis virus internal ribosome entry site (EMCV IRES), the GFP reporter gene and the SV40 PolyA sequences, all within RL1 flanking sequences. This novel arrangement of DNA sequences or 'smart cassette' allows ICP34.5 null HSV-1,

expressing a gene of interest in the RL1 locus, to be easily generated by simply inserting the desired transgene (downstream of a suitable promoter) into the MCS and co-transfecting BHK cells with the plasmid and HSV-1 DNA. The IRES situated between the GFP gene and the MCS permits expression of two genes from the same promoter and so recombinant virus expressing the gene of interest also expresses GFP and can therefore be easily identified under a fluorescence microscope and purified.

Materials and Methods

1μg of RL1.del* was digested with 10units *Hpa*I (Promega) in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega) at 37°C for 16hrs. The digested plasmid was then purified using the QIAquick PCR purification kit (Qiagen), treated with 10 units of Calf Intestinal Phosphatase (Promega), in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using a Qiaquick PCR purification kit. 5μl of the purified DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 2).

4 x 1μg of pNAT-IRES-GFP** was digested with 10 units of *Nsi*I and 10 units of *Ssp*I in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega) at 37°C for 16hrs. The reaction mixture was electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 5.4Kbp DNA fragment consisting of the CMV IE promoter (pCMV), upstream of the noradrenaline transporter gene (NAT), the encephalomyocarditis virus internal ribosome entry site (IRES), the gene for green fluorescent protein (GFP) and

the SV40 polyadenylation sequences (SV40 Poly A), was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit (Qiagen). The eluted DNA was blunt ended using 3 units Klenow Polymerase (Promega) in accordance with the manufacturers instructions and the DNA purified using a QIAquick PCR purification kit (Qiagen). 5µl of the purified DNA fragment was electrophoresed on a 1% agarose gel to check its concentration (Figure 3).

Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the *Hpa*I digested/CIP treated RL1.del and blunt ended pCMV-NAT-IRES-GFP-SV40 Poly A DNA, at 16^{0}C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliqouts of the ligation reactions***. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using *Afl*II (New England Biolabs) restriction enzyme analysis. Plasmid DNA containing the insert would produce two fragments of 4.8Kbp and 9.2Kbp following digestion with *Afl*II. Two clones (clone 5 and 8) contained the insert (Figure 4). The orientation of the insert in clone 5 (RL1.dCMV-NAT-GFP) was determined using *Xho*I restriction enzyme analysis (Figure 5).

To generate RL1.dIRES-GFP from clone 5, the CMV-NAT portion of the CMV-NAT-IRES-GFP-SV40 PolyA insert was removed by digesting 4 x 500ng of clone 5 with 10 units of *Xho*I in a suitable volume of buffer and water (Promega), overnight at 37^{0}C. The digested DNA was

electrophoresed on a 1% agarose gel at 110 volts for 1hr (Figure 6A). The 10.2Kbp fragment consisting of the IRES, the GFP gene, the SV40 PolyA sequences and RL1 flanking sequences in a pGEM3Zf(-) (Promega) backbone, was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit.

Ligation reactions were performed in small eppendorf tubes containing 100ng - 500ng purified DNA, 3 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega) and nuclease free water (Promega) overnight at 16^0C. Competent JM109 bacterial cells (Promega) were then transformed with various aliquots of the ligation reactions***. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened using *Xho*I (Promega) restriction enzyme analysis. Colonies containing plasmid DNA from which CMV-NAT had been removed would produce one fragment of 10.2Kbp when digested with *Xho*I. Several positive clones were found, one was isolated, and a large-scale plasmid preparation undertaken using Promega's Wizard Plus Maxipreps kit. The large-scale plasmid preparation was checked by digesting with *Xho*I (Figure 6B). This plasmid DNA was subsequently named 'RL1.dIRES-GFP'.

Plasmid RL1.dIRES-GFP has been deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG, United

Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty.

RL1.del

*RL1.del was provided by Dr.E.McKie and is the pGEM-3Zf(-) plasmid (Promega) into which has been cloned an HSV-1 fragment (123459-129403) consisting of the RL1 gene and its flanking sequences. The 477bp *Pfl*MI-*Bst*EII fragment of the RL1 gene (125292-125769) has been removed and replaced with a multi-cloning site (MCS) to form RL1.del.

pNAT-IRES-GFP

** pNAT-IRES-GFP was supplied by Dr. Marie Boyd (CRUK Beatson Laboratories) and is the pIRES2-EGFP plasmid (BD Biosciences Clontech) into which she has cloned the bovine noradrenaline transporter (NAT) gene (3.2Kbp), at the *Nhe*I and *Xho*I sites.

****Transformation of Bacterial Cells*

10µl of a glycerol *E.coli* stock was added to 10ml 2YT medium in a 20ml griener tube. This was placed in a 37°C shaking incubator for 16-24hrs until a saturated culture was obtained. 1ml of this culture was then added to 100ml of 2YT in a 500ml sterile glass bottle and placed in the 37°C shaking incubator for 3hrs. The bacterial cells were pelleted by centrifugation at 2,000rpm for 10 minutes (Beckman). The cells were then resuspended in 1/10th volume of transformation and storage buffer (10mM $MgCl_2$, 10mM $Mg(SO)_4$, 10% (w/v) PEG 3,500, 5% (v/v) DMSO). The cells were placed on ice for between 10 minutes and 2hrs, after which time they were considered competent for transformation.

1-10µl of DNA was mixed with 100µl of competent bacteria in eppendorf tubes, and the tubes placed on ice for 30 minutes. After this, the samples were 'heat shocked' by incubating the tubes in a 42°C water bath for exactly 45 seconds before placing them on ice for a further 2 minutes. 1ml of L-Broth was added, the tube inverted 2-3 times, and the bacteria incubated for 1hr at 37°C. 100µl of the transformed bacteria was plated out onto L-broth agar plates containing 100µg/ml of the appropriate antibiotic (usually ampicillin or kanamycin). Plates were allowed to dry at room temperature, before incubating in an inverted position at 37°C overnight.

Example 2

Generation of ICP34.5 null HSV-1 expressing a gene product of interest and GFP using plasmid RL1.dIRES-GFP.

General Approach

Generation of ICP34.5 null HSV-1 expressing a gene product of interest requires insertion of nucleotide sequence encoding the gene product (polypeptide) of interest and desired promoter at the MCS of RL1.dIRES.GFP followed by co-transfection of BHK cells with the linearised plasmid, containing the gene of interest, and HSV DNA. Following homologous recombination viral plaques expressing GFP are identified. Figure 7 illustrates the method steps involved.

Referring to Figure 7A plasmid DNA, containing the gene of interest and the desired promoter (X), is digested

with restriction endonucleases to release the promoter/gene fragment.

The promoter/gene fragment is purified and cloned into the multi-cloning site (MCS) of RL1.dIRES.GFP forming a shuttle vector suitable for generating oncolytic HSV-1 (Figure 7B). This vector contains HSV-1 sequences that flank the essential RL1 gene but does not contain the RL1 gene. The plasmid also contains the gene for Green Fluorescent Protein (GFP) downstream of an internal ribosome entry site (IRES). The IRES permits expression of both the gene of interest and the GFP gene from the same upstream promoter.

BHK cells are then co-transfected with linearised RL1.dIRES.GFP, now containing the gene of interest, and HSV-1 DNA (Figure 7C). Following homologous recombination, designer virus, expresssing the gene of interest and GFP, is generated and can be distinguished from wild type virus (also generated but not expressing GFP) under a fluorescence microscope.

Viral plaques, expressing GFP (and hence the gene of interest), are picked under the fluorescence microscope and purified until all wild-type HSV-1 has been removed. The recombinant HSV-1 is considered 100% pure when all the viral plaques are expressing GFP (Figure 7D).

Once the recombinant virus is completely pure, an isolated plaque is picked and a highly concentrated stock is grown and titrated (Figure 7E). Oncolytic HSV-1, expressing a gene product of interest from a selected

promoter, is then ready for characterisation and in vitro examination of its tumour killing potential.

<u>Materials and Methods</u>

To generate recombinant ICP34.5 null HSV-1 expressing a gene of interest and GFP, requires the gene of interest and a suitable promoter to be cloned into the MCS of RL1.dIRES-GFP in the forward orientation with respect to the GFP gene in this plasmid. Once this has been achieved the plasmid is linearised (i.e. digested with a restriction enzyme that cuts only once, usually *Ssp*I or *Sca*I) in an irrelevant region. 80% confluent BHK cells in 60 mm petri dishes are then co-transfected with HSV-1 DNA and linearised plasmid DNA as described below.

To generate replication restricted HSV-1, expressing the gene of interest and GFP, the gene of interest must be cloned into RL1dIRES-GFP downstream of a suitable promoter (e.g. CMV IE). The promoter is required upstream of the gene of interest for the production of a bicistronic mRNA transcript. The IRES sequence between the two open reading frames in the transcript functions as a ribosome binding site for efficient cap-independent internal initiation of translation. The design enables coupled transcription of both the gene of interest and GFP, followed by cap-dependent initiation of translation of the first gene (gene of interest) and IRES-directed, cap-independent translation of GFP. Co-ordinate gene expression is thus ensured in this configuration.

Co-Transfection of Virus and Plasmid DNA by $CaPO_4$ and DMSO Boost

HSV-1 (17^+) DNA and 0.1-1µg linearized SMART cassette containing the gene and promoter of interest is pipetted into 1.5ml eppendorf tubes containing 1µl of calf thymus DNA (10µg/ml) and an appropriate volume of distilled water to give a final volume of 165µl. The solutions are very gently mixed using a 200µl pipette tip. 388µl of HEBS, pH 7.5, (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM HEPES) is then added, the solution mixed, before adding 26.5µl of 2M $CaCl_2$ dropwise and flicking the eppendorf tube two or three times. The samples are left at room temperature for 10-15 minutes then added dropwise to 80% confluent BHK's in 60mm petri dishes from which the medium has been removed. Following incubation at 37°C for 45 minutes, the cells are overlaid with 5ml of ETC10 and incubated at 37°C. Three to four hours later, the media is removed and the plates washed with ETC10. For exactly 4 minutes, the cells are overlaid with 1ml 25% (v/v) DMSO in HEBS at room temperature. After the 4 minutes, the cells are immediately washed three times with 5ml ETC10 before overlaying with 5ml of ETC10 and returning to the incubator. The following day, fresh medium is added to the cells. Two days later, when cpe is evident, cells are scraped into the medium, transferred to small bijoux and sonicated thoroughly. The sample is then stored at −70°C until required (see section below on plaque purification).

N.B. The volume of virus DNA to add is determined by undertaking the above procedure without plasmid DNA, using a range of virus DNA volumes and choosing the volume that gives the greatest number of viral plaques on the BHK monolayer after 2 or 3 days.

Plaque Purification

Sonicated samples from co-transfection plates are thawed and serially diluted 10 fold in ETC10. 100µl from neat to the 10^5 dilution is plated out on confluent BHK's in 60 mm petri dishes from which the media has been removed. After 45 minutes incubation at 37°C, the cells are overlaid with 5ml EMC10 and incubated at 37°C for 48hrs. The plates are then checked for the presence of viral plaques and those dishes with the fewest, most separated plaques are placed under a fluorescent stereomicroscope. Recombinant virus, designed to express the green fluorescent protein (GFP) in addition to the gene of interest, can clearly be distinguished from wild type virus using a GFP filter. Fluorescent plaques are picked using a 20µl pipette and placed (including the tip) into an eppendorf tube containing 1ml ETC10. The sample is thoroughly sonicated before making serial 10 fold dilutions in ETC10 and repeating the above purification procedure. The process is repeated typically 3-4 times until every plaque on the BHK monolayer is fluorescent. Once this has been achieved, 50µl of this sample is used to infect BHK's in roller bottles, in 50ml ETC10, and a virus stock grown.

Tissue Culture Media

BHK21/C13 cells are grown in Eagle's medium (Gibco) supplemented with 10% newborn calf serum (Gibco) and 10% (v/v) tryptose phosphate broth. This is referred to as ETC10. For virus titrations and plaque purification, EMC10 (Eagles medium containing 1.5% methylcellulose and 10% newborn calf serum) is used to overlay the cells.

Example 3

Construction of HSV1716/CMV-NTR/GFP

General Approach

HSV1716/CMV-NTR/GFP was generated by cloning a 1.6Kbp *Bam*HI fragment from pPS949[10], consisting of the *E.coli* nitroreductase (NTR) gene downstream of the CMV IE promoter (pCMV), into the MCS of the RL1.dIRES-GFP smart cassette, in the forward orientation with respect to the GFP gene in RL1.dIRES-GFP (Figure 8). The resultant plasmid, named RL1.dCMV-NTR-GFP, was then linearised and recombinant virus generated and purified as described above. The plasmid pPS949 (referred to as 'pxLNC-ntr' in Ref 10) containing the NTR gene downstream of the CMV IE promoter (pCMV-NTR) in a pLNCX (Clontech) backbone, was a kind gift from Professor Lawrence Young, University of Birmingham.

Materials and Methods

4 x 1μg of pPS949 was digested with 10 units of *Bam*HI (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. The reaction mixture was electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 1.6Kbp DNA fragment consisting of the CMV promoter upstream of the NTR gene (pCMV-NTR), was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit (Qiagen). 5μl of the purified DNA fragment was

electrophoresed on a 1% agarose gel to check its concentration (Figure 9).

2μg of the RL1.dIRES-GFP smart cassette was then digested with 15 units of *Bgl*II (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. The digested plasmid was then purified using the QIAquick PCR purification kit (Qiagen), treated with 10 units of Calf Intestinal Phosphatase (Promega), in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using the Qiaquick PCR purification kit. 5μl of the purified DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 10).

Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the *Bgl*II digested/CIP treated RL1.dIRES-GFP smart cassette and pCMV-NTR (*Bam*HI ends), at 16°C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliqouts of the ligation reactions. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using *Bgl*II/*Xho*I (Promega) restriction enzyme analysis. RL1.dIRES-GFP plasmid DNA containing the pCMV-NTR insert in the correct orientation would produce two fragments of 11.5Kbp and 300bp following digestion with *Bgl*II and *Xho*I. One clone (clone 4) was found to contain the insert in the correct orientation (Figure 11). This plasmid was named 'RL1.dCMV-NTR-GFP'.

0.1-1μg of RL1.dCMV-NTR-GFP was linearized by digesting with 10 units of *Sca*I (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37^0C for 16hrs. A sample (5μl) of the digested DNA was electrophoresed on a 1% agarose gel for 1hr at 110 volts to check that it had been linearized. 80% confluent BHK cells were then co-transfected with a suitable volume of the remaining linearised DNA and HSV-1 DNA. Recombinant HSV-1, expressing GFP (and hence NTR), was identified and purified using a fluorescent microscope and a virus stock, named HSV1716/CMV-NTR/GFP, was grown and titrated on BHK cells (Figure 12).

HSV1716/CMV-NTR/GFP has been deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 05 November 2003 at the European Collection of Cell Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03110501 in accordance with the provisions of the Budapest Treaty.

<u>HSV1716/CMV-NTR/GFP Cell Killing</u>

HSV1716/CMV-NTR/GFP replicates with almost identical kinetics to HSV1716 in BHK cells and 3T6 cells. BHK cells support the replication of ICP34.5 null HSV while confluent 3T6 cells do not. Figure 13 shows that HSV1716/CMV-NTR/GFP will replicate as well as HSV1716 in permissive cell lines and that the introduction of exogenous genes, e.g. NTR and GFP, has not reduced the oncolytic potential of the ICP34.5 null HSV. The fact that HSV1716/CMV-NTR/GFP fails to replicate in 3T6 cells

also indicates that this recombinant HSV is an ICP34.5 null mutant.

Figure 14 is a Western blot demonstrating that no ICP34.5 polypeptide is expressed from HSV1716/CMV-NTR/GFP, and that the virus is thus useful as a gene therapy vector.

Figure 15 is another Western blot demonstrating expression of NTR in a variety of cell lines infected with HSV1716/CMV-NTR/GFP, including a human malignant melanoma cell line (C8161) and confluent 3T6 cells in which ICP34.5 null HSV does not replicate. Expression of NTR in confluent 3T6 cells, following infection with HSV1716/CMV-NTR/GFP, is encouraging as it demonstrates that replication of this ICP34.5 null mutant is not required for expression of the prodrug-activating gene (i.e. NTR). Some tumour cells *in vivo* will not support the replication of ICP34.5 null HSV and as such, will not be killed with HSV1716.

Figure 16 shows the results from a cytotoxicity assay performed in confluent 3T6 cells. Infecting confluent 3T6 cells with an ICP34.5 null mutant (HSV1716/CMV-NTR/GFP), at a multiplicity of infection (MOI) of 1 plaque forming units (pfu)/cell, does not result in any significant cell death, neither does separate incubation of the cells with 50μM CB1954. However, significant cell death is evident 72hrs post infection with 1pfu/cell HSV1716/CMV-NTR/GFP when 50μM CB1954 is included in the growth medium. This clearly demonstrates that when there is no replication of the virus, substantial cell death is still possible from virus directed enzyme prodrug therapy (VDEPT).

Infecting confluent 3T6 cells with an ICP34.5 null mutant at a MOI of 10pfu/cell will result in cell death, by a mechanism known as 'viral antigen overload'. However, the level of cell killing is even more pronounced (approximately 20% more), when 50μM CB1954 is included in the growth medium.

A similar cytotoxicity assay was performed in human C8161 melanoma cells, the results are set out in Figure 17. Unlike confluent 3T6 cells, C8161 cells do support the replication of ICP34.5 null HSV. Therefore, cell death will occur following infection of the cells with ICP34.5 null HSV, at 1pfu/cell. However, when CB1954 is included in the overlay of HSV1716/CMV-NTR/GFP infected cells, the cells are killed more efficiently and more quickly. No enhanced cell killing is evident when CB1954 is included in the overlay of cells infected with HSV1716-GFP. These results demonstrate that enhanced cell killing is possible in human tumour cells.

Cell culture images for the cytotoxicity assays performed in confluent 3T6 and human C8161 melanoma cells are shown in Figures 18 and 19.

Example 4

Construction of HSV1716/CMV-asSCCRO/GFP

General Approach

HSV1716/CMV-asSCCRO/GFP was generated by first digesting pUSEamp-asSCCRO with *Ssp*I and *Xho*I and purifying the 1.96Kbp fragment generated from the digestion. The

1.96kbp *Ssp*I/*Xho*I fragment comprises DNA antisense to squamous cell carcinoma related antigen (asSCCRO), downstream of the CMV IE promoter (pCMV). This fragment was cloned into the MCS of the RL1.dIRES-GFP smart cassette, in the forward orientation with respect to the GFP gene in RL1.dIRES-GFP (Figure 20). The resultant plasmid, named RL1.dCMV-asSCCRO-GFP, was then linearised and recombinant virus generated and purified as described in Example 2. The plasmid pUSEamp-asSCCRO was obtained from Bhuvanesh Singh, Memorial Sloan Kettering Cancer Center, New York.

Materials and Methods

2µg of the RL1.dIRES-GFP plasmid was then digested with 15 units of *Bgl*II (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. The digested plasmid was then purified using the QIAquick PCR purification kit (Qiagen), treated with 5 units of Klenow polymerase (Promega) for 20 minutes at room temperature, then purified again. The purified DNA was then added to 10 units of Calf Intestinal Phosphatase (Promega), in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using the QIAquick PCR purification kit. 5µl of the purified DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 21).

4 x 1µg of pUSEamp-asSCCRO was digested with 10 units of *Ssp*I and 10 units of *Xho*I (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. The reaction mixture was

electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 1.96Kbp DNA fragment, consisting essentially of the CMV promoter upstream of DNA antisense to SCCRO (pCMV-asSCCRO), was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit (Qiagen). The purified DNA was blunt ended using 5 units of Klenow polymerase (Promega) for 20 minutes at room temperature, then purified again. 5µl of the purified DNA fragment was electrophoresed on a 1% agarose gel to check its concentration (Figure 22).

Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the *Bgl*II digested/blunt ended/CIP treated RL1.dIRES-GFP plasmid and blunt ended pCMV-asSCCRO, at 16°C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliqouts of the ligation reactions. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using *Bgl*II (Promega) restriction enzyme analysis. RL1.dIRES-GFP plasmid DNA containing the pCMV-asSCCRO insert would produce two fragments of 10.8Kbp and 1.4Kbp following digestion with *Bgl*II. One clone (clone 11) was found to contain the insert (Figure 23). The pCMV-asSCCRO insert could have been cloned into RL1.dIRES-GFP in two orientations. To confirm that the pCMV-asSCCRO fragment had been cloned into RL1.dIRES-GFP in the desired orientation, clone 11 was digested with 10 units of *Nru*I (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. If the insert was in

the correct orientation, a fragment of 1.64Kbp would be generated. As a 1.64Kbp fragment was generated following digestion with *Nru*I (Figure 24), it was confirmed that pCMV-asSCCRO had been cloned in the desired orientation. This plasmid (clone 11) was named 'RL1.dCMV-asSCCRO-GFP'.

0.1-1μg of RL1.dCMV-asSCCRO-GFP was linearized by digesting with 10 units of *Sca*I (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. A sample (5μl) of the digested DNA was electrophoresed on a 1% agarose gel for 1hr at 110 volts to check that it had been linearized. 80% confluent BHK cells were then co-transfected with a suitable volume of the remaining linearised DNA and HSV-1 DNA. Recombinant HSV-1, expressing GFP (and hence asSCCRO), was identified and purified using a fluorescent microscope and a virus stock, named HSV1716/CMV-asSCCRO/GFP, was grown and titrated on BHK cells (Figure 25).

Example 5

The use of HSV1716asSCCRO as a novel therapeutic agent for head and neck squamous cell cancer.

Squamous cell carcinoma of the head and neck afflicts an estimated 125,000 patients annually in Europe, North America and the Far East. Primary therapy for localized disease is surgery and adjuvant radiotherapy. Tumours recur in approximately one-third of patients. Once the cancer has recurred and/or metastasized, the patient is considered incurable. Combination chemotherapy induces responses in 30-50% of patients but there is no clear

impact on survival. There remains an urgent need for more effective therapies [12,13]. There has been much interest in the use of novel therapies in this disease with particular focus on oncolytic viruses by direct intratumoural injection. The use of oncolytic viruses to selectively kill tumours while leaving normal cells unaffected is a very attractive concept as it has the potential to limit the toxicity which occurs with conventional modalities. Recent research has been carried out using intratumoural injections of a selectively replicating adenovirus (Onyx-015) for the local control of recurrent disease. Phase I/II studies involving virus alone and in combination with chemotherapy have produced encouraging results [14,15,16]. Selectively replicating Herpes simplex viruses HSV may have better efficacy due to its more potent replication and oncolytic potential. HSV1716[17] is a deletion mutant of herpes simplex virus which fails to synthesise the virulence protein ICP34.5. It has been shown that HSV1716 replicates in actively dividing cells but not in resting or terminally differentiated cells[18,19]. In vivo, HSV1716 administration has been carried out in mouse models of a range of cancers including melanoma, teratocarcinoma, glioma, medulloblastoma and mesothelioma. Animals showed improved survival and tumour regression following administration of HSV1716 [20,21,22,23,24,25] with no evidence of replication in normal tissue and no toxicity. HSV1716 has been used in Phase 1 trials in patients with glioblastoma multeforme (GBM)[26], melanoma and head and neck cancer. No toxicity has been experienced and patients who were seropositive pre HSV1716 seroconverted and evidence of virus replication contained within tumours has been obtained.

It has been shown that the novel oncogene SCCRO (Squamous cell carcinoma related oncogene) is amplified in 30% of mucosal squamous cell cancers and that overexpression is associated with poor prognosis in head and neck cancer patients. The inventors believe that insertion of the antisense to SCCRO into the herpes simplex virus HSV1716 will produce a virus with a dual hit mechanism of cell kill. This would involve virus induced cell death via cytolysis in addition to cell death via downregulation of endogenous SCCRO expression.

The HSV1716asSCCRO virus was constructed, amplified and purified in accordance with the present invention. Following this, in vitro and in vivo analysis was carried out on a series of head and neck squamous cell cancer (HNSCC) cell lines. HNSCC cell lines studied were SCC15, 1483, 1186, 1386, 1986 and 584. The relative expression of SCCRO protein expression in these cell lines was initially determined by western blotting. This showed the cell lines SCC15, 1483 and 1186 had high levels of expression of SCCRO, 1386 intermediate expression and 1986, 584 low expression. All cell lines were then infected with HSV1716 or HSV1716asSCCRO viruses and cytotoxicity determined by LDH release cytotoxicity assay at MOI (multiplicity of infection) of 1 and 5pfu/cell (Figures 26, 27 and 28). Viral proliferation was determined by serial plaque assays at an MOI of 1pfu/cell (Figure 29) and infectivity determined by green fluorescent protein (gfp) using HSV1716gtp virus (Figure 30). In the cell lines with low or intermediate expression (1986,584,1386) cytotoxicity increased in a dose dependent fashion with both viruses but there was no

significant difference in cytotoxicity between the 2 viruses. Viral proliferation assays (Figure 29) showed an increase in viral production over a range of 10^2 to 10^4 with equivalent proliferation with both viruses. In the cell lines with high expression of SCCRO the inventors found that the cell line SCC15 showed enhanced cytotoxicity with the HSV1716asSCCRO virus. This observation occurred 12 hours post viral infection which is premature for virus induced cell death by a cytolytic mechanism. In addition, virus proliferation of the 2 viruses was equivalent with an increase in virus production of 10^4 for both viruses. These results suggested that the enhanced cell kill at 12 hours was by an alternative mechanism possibly by downregulation of the endogenous high expression of SCCRO by antisenseSCCRO expression. To investigate this hypothesis the inventors analysed the cell lines SCC15 (high expression) and 584 (low expression) post virus infection by serial protein expression over a 36 hour period. Cells were infected at an MOI of 1pfu/cell with HSV1716 or HSV1716asSCCRO and cells harvested and lysed for protein at 12, 24 and 36 hours post infection. Western blotting of the cell line SCC15 showed downregulation of SCCRO protein at 12 hours with HSV1716asSCCRO but not in 584 (see Figure 31). This suggested that this was the mechanism by which HSV1716asSCCRO had enhanced efficacy in cell line SCC15.

In vivo studies were then carried out in the cell lines SCC15 and 584. Subcutaneous tumour were grown in athymic nude mice and injected intratumorally with a single injection of HSV1716, HSV1716asSCCRO or PBS control and tumour sizes monitored at serial time points (Figure 32 and 33). In SCC15, efficacy was enhanced with

HSV1716asSCCRO compared to HSV1716. All 6 mice injected with HSV1716asSCCRO showed complete responses by 21 days post infection. Inhibition of tumour growth occurred with HSV1716 with only 3/6 mice showing a complete response over a 48 day follow up period. In the cell line 584, both viruses were able to inhibit tumour growth but neither virus produced a complete response in any mouse xenograft injected. This in vivo data was further evidence that HSV1716asSCCRO was a more potent antitumour agent than HSV1716 in the cell line SCC15 with high SCCRO expression.

These results suggest that HSV1716 and HSV1716asSCCRO has great potential as useful therapeutic agents in the treatment of recurrent or locally advanced head and neck cancer by direct intratumoral injection. However, this data also suggests that HSV1716asSCCRO may augment anti-tumour activity in SCCRO over-expressing tumours. Since SCCRO is overexpressed in a significant number of squamous cell cancers of the head and neck this modified virus may be particularly efficacious in this disease. Therefore, the inventors believe that HSV1716asSCCRO will be an important therapeutic agent in head and neck cancer patients with locally advanced or recurrent head and neck cancer, particularly as these cancers are amenable to direct intratumoural injection.

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26. Rampling R, Cruickshank G, Papanastassiou V, Nicoll J, Hadley D, Petty R, Maclean A, Harland J, McKie E, Mabbs R. & Brown SM. (2000). Toxicity evaluation of replication competent herpes simplex virus (ICP 34.5

null mutant 1716) in patients with recurrent malignant glioma. *Gene Therapy* 7, 859-866.

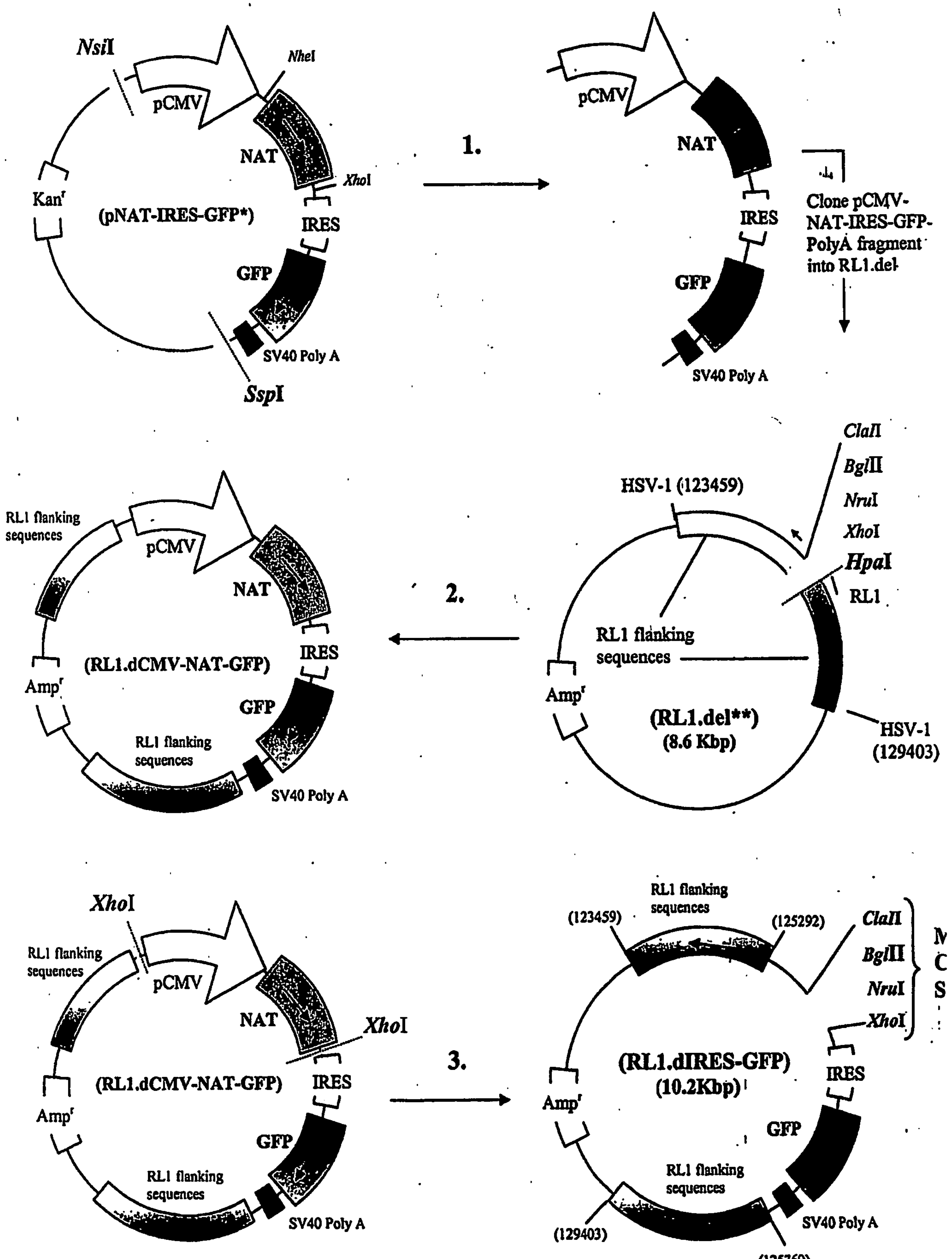

NsiI
NheI
pCMV
NAT
XhoI
Kan^r
(pNAT-IRES-GFP*)
IRES
GFP
SV40 Poly A
SspI
1.
pCMV
NAT
IRES
GFP
SV40 Poly A
Clone pCMV-NAT-IRES-GFP-PolyA fragment into RL1.del
ClaII
BglII
NruI
XhoI
HpaI
HSV-1 (123459)
RL1
RL1 flanking sequences
Amp^r
(RL1.del**)
(8.6 Kbp)
HSV-1 (129403)
2.
RL1 flanking sequences
pCMV
NAT
IRES
Amp^r
(RL1.dCMV-NAT-GFP)
GFP
RL1 flanking sequences
SV40 Poly A
XhoI
RL1 flanking sequences
pCMV
NAT
XhoI
Amp^r
(RL1.dCMV-NAT-GFP)
IRES
GFP
RL1 flanking sequences
SV40 Poly A
3.
RL1 flanking sequences
(123459)
(125292)
ClaII
BglII
NruI
XhoI
MCS
Amp^r
(RL1.dIRES-GFP)
(10.2Kbp)
IRES
GFP
RL1 flanking sequences
SV40 Poly A
(129403)
(125769)

Figure 1

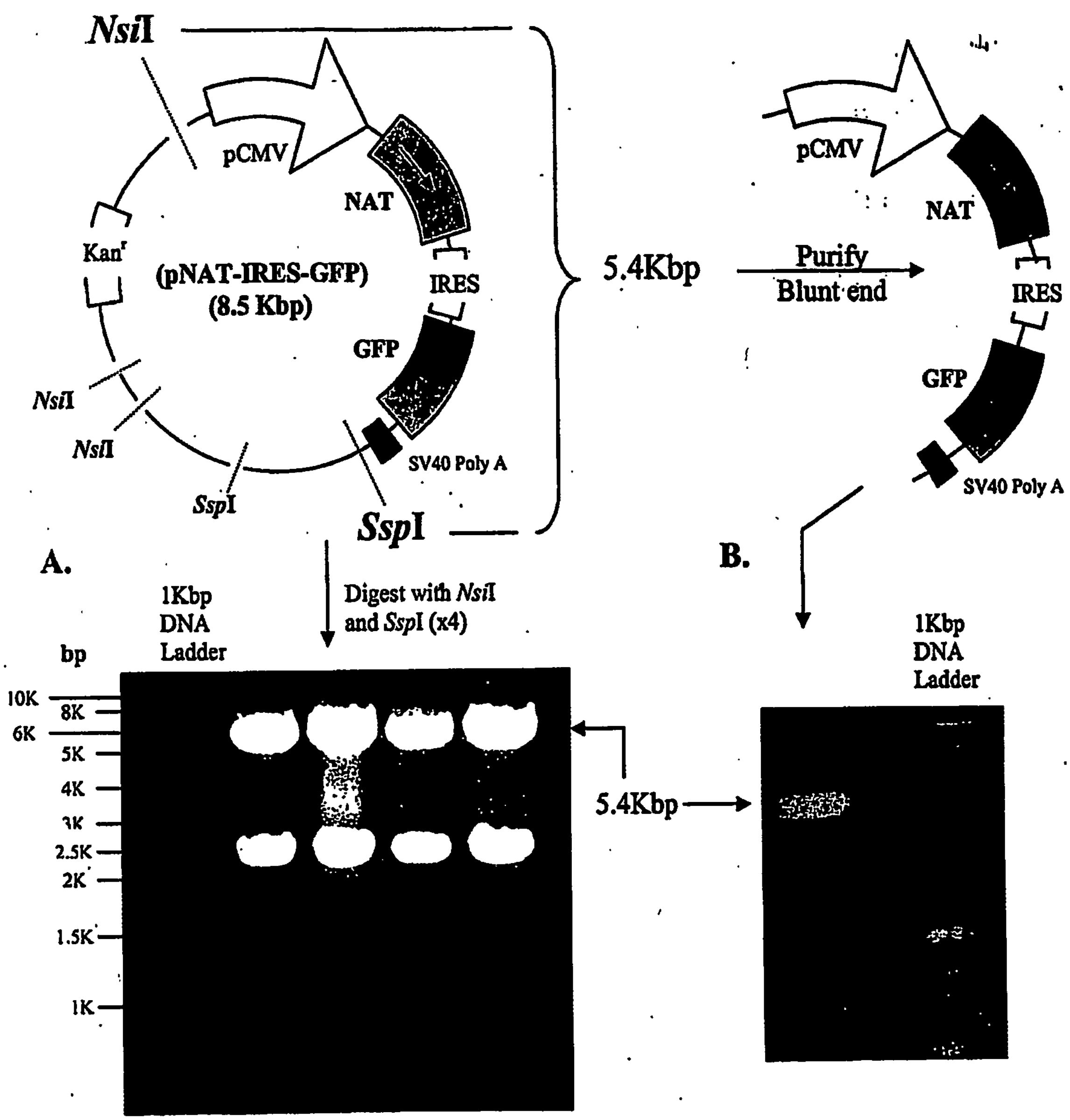

NsiI
pCMV
NAT
Kan^r
(pNAT-IRES-GFP)
(8.5 Kbp)
IRES
GFP
NsiI
NsiI
SspI
SV40 Poly A
SspI
5.4Kbp
Purify
Blunt end
pCMV
NAT
IRES
GFP
SV40 Poly A
A.
B.
1Kbp DNA Ladder
Digest with NsiI and SspI (x4)
bp
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K
1K
5.4Kbp
1Kbp DNA Ladder

Figure 3

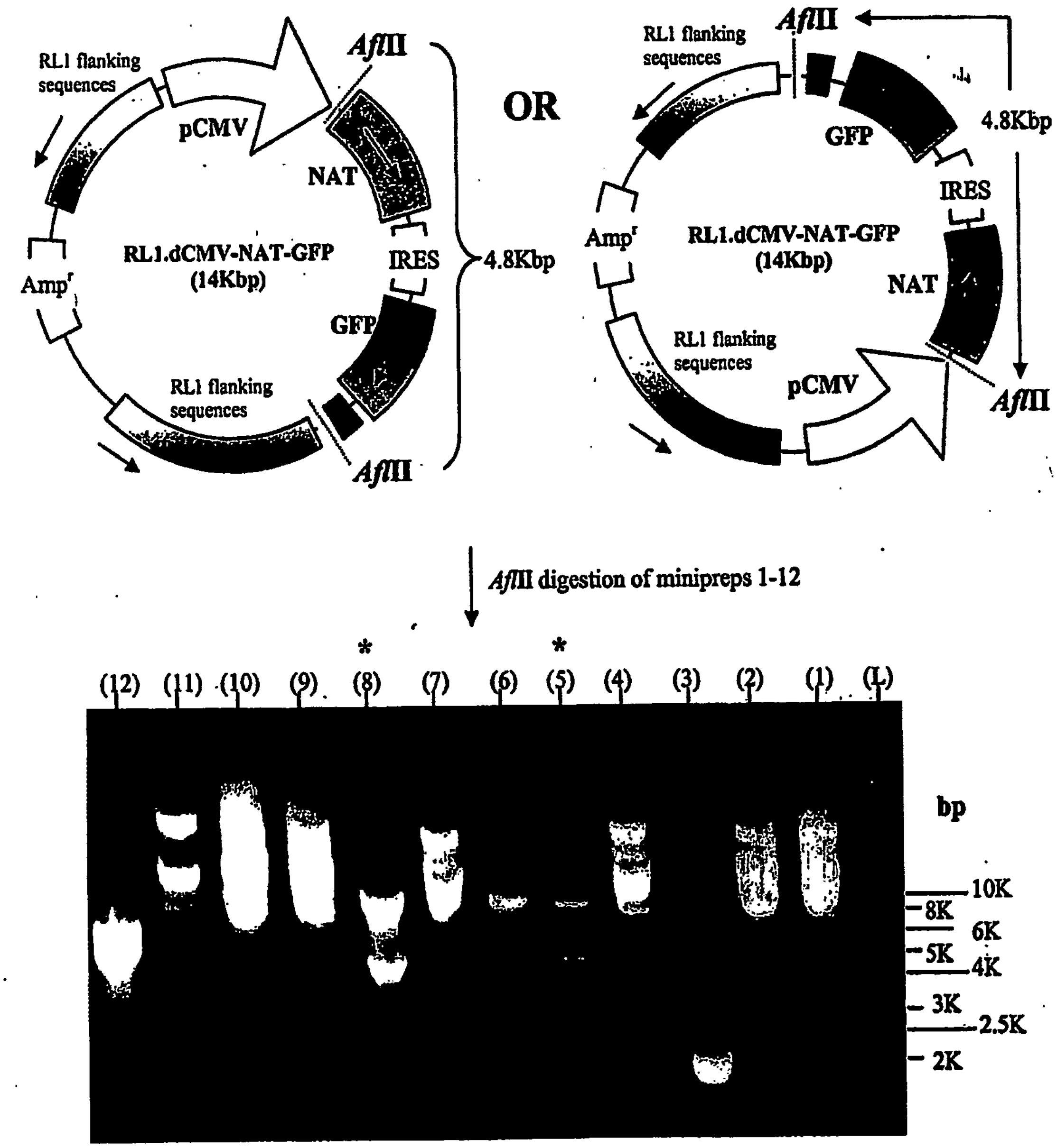
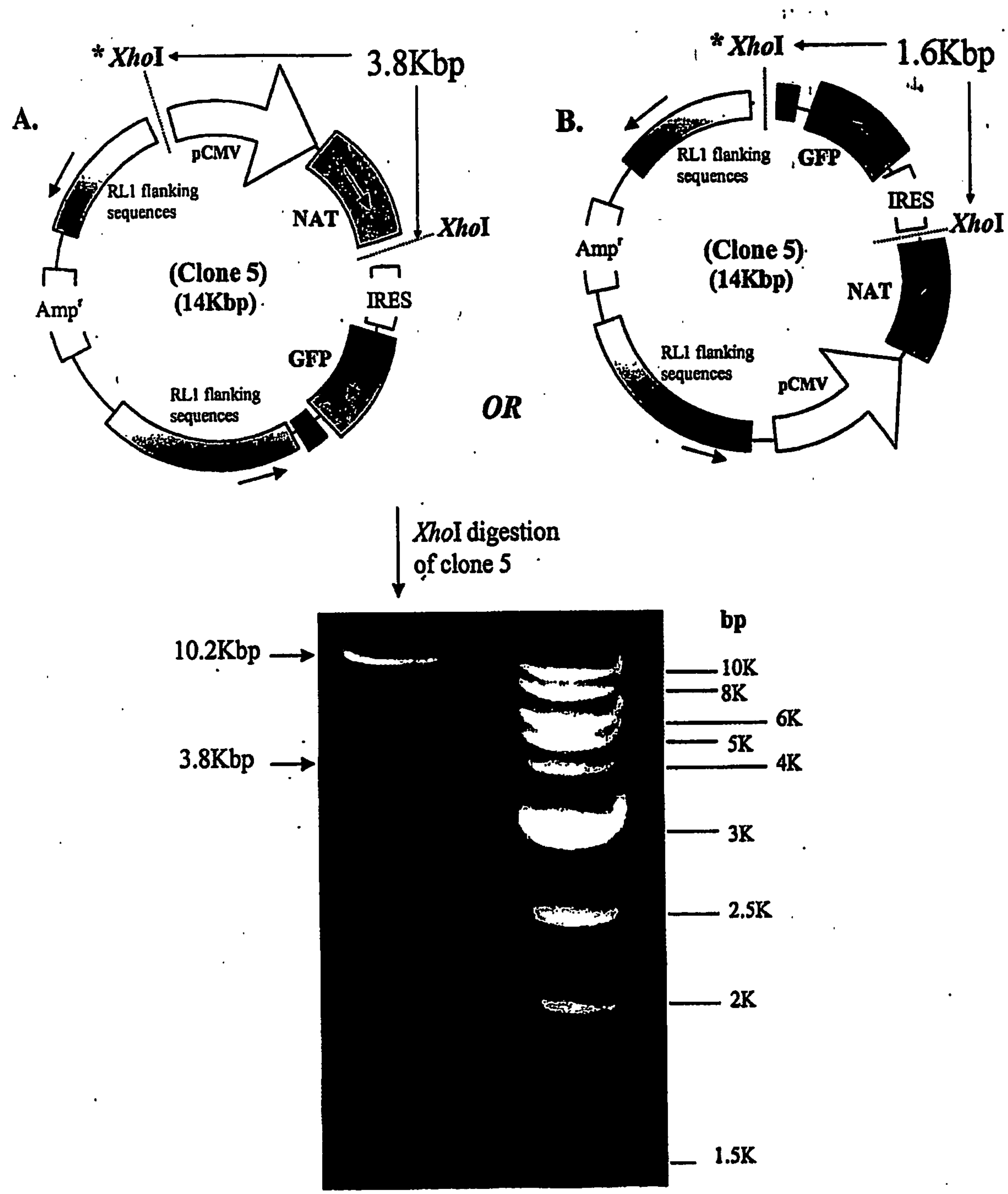

RL1 flanking sequences
pCMV
AflII
NAT
IRES
GFP
RL1.dCMV-NAT-GFP
(14Kbp)
Amp^r
RL1 flanking sequences
AflII
4.8Kbp
OR
RL1 flanking sequences
AflII
4.8Kbp
GFP
IRES
Amp^r
RL1.dCMV-NAT-GFP
(14Kbp)
NAT
RL1 flanking sequences
pCMV
AflII
AflII digestion of minipreps 1-12
*
*
(12) (11) (10) (9) (8) (7) (6) (5) (4) (3) (2) (1) (L)
bp
10K
8K
6K
5K
4K
3K
2.5K
2K

Figure 4



A.
*XhoI
3.8Kbp
pCMV
RL1 flanking sequences
NAT
XhoI
(Clone 5)
(14Kbp)
Amp^r
IRES
GFP
RL1 flanking sequences
OR
B.
*XhoI
1.6Kbp
RL1 flanking sequences
GFP
IRES
XhoI
Amp^r
(Clone 5)
(14Kbp)
NAT
RL1 flanking sequences
pCMV
XhoI digestion of clone 5
10.2Kbp
3.8Kbp
bp
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K

Figure 5

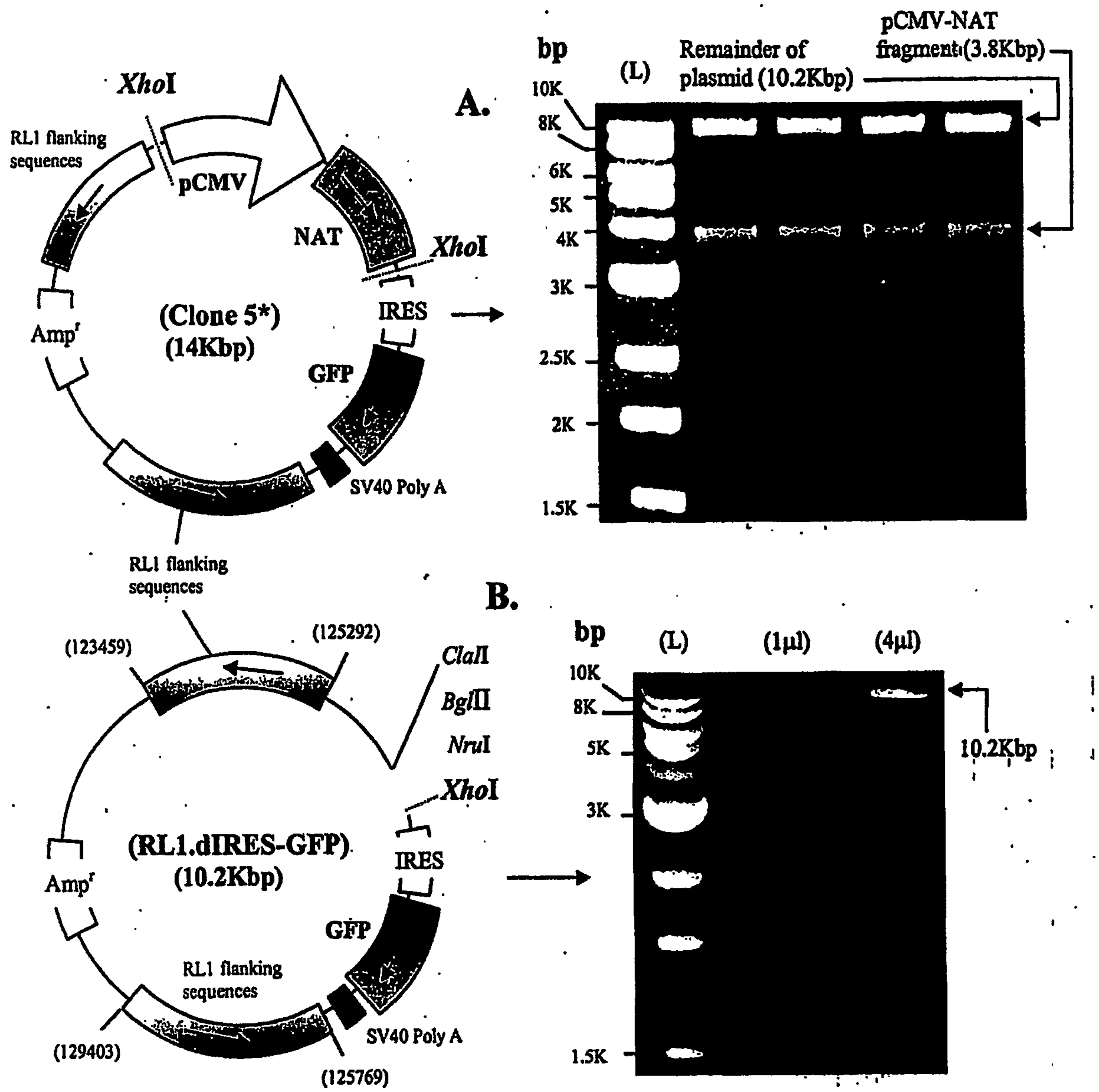
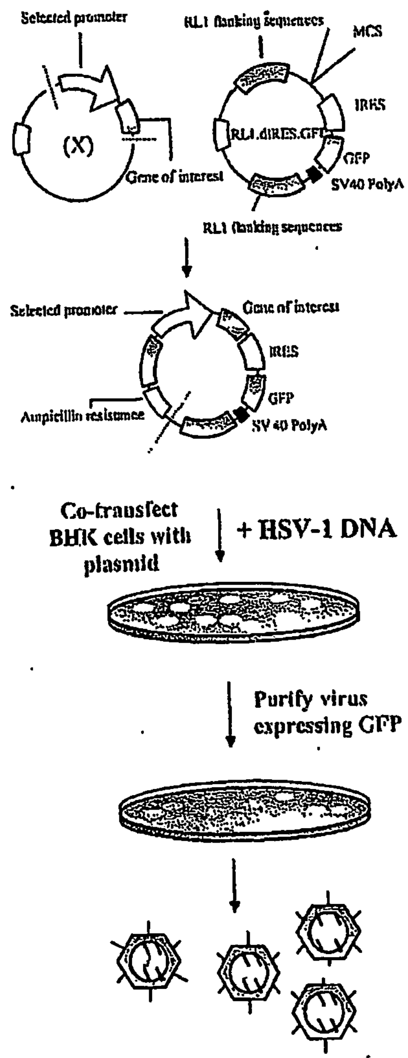

A.
XhoI
RL1 flanking sequences
pCMV
NAT
XhoI
IRES
(Clone 5*)
(14Kbp)
Amp^r
GFP
SV40 Poly A
RL1 flanking sequences
bp
(L)
Remainder of plasmid (10.2Kbp)
pCMV-NAT fragment (3.8Kbp)
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K
B.
(123459)
(125292)
ClaI
BglII
NruI
XhoI
(RL1.dIRES-GFP)
(10.2Kbp)
Amp^r
IRES
GFP
RL1 flanking sequences
SV40 Poly A
(129403)
(125769)
bp
(L)
(1µl)
(4µl)
10K
8K
5K
3K
1.5K
10.2Kbp

Figure 6



A

B

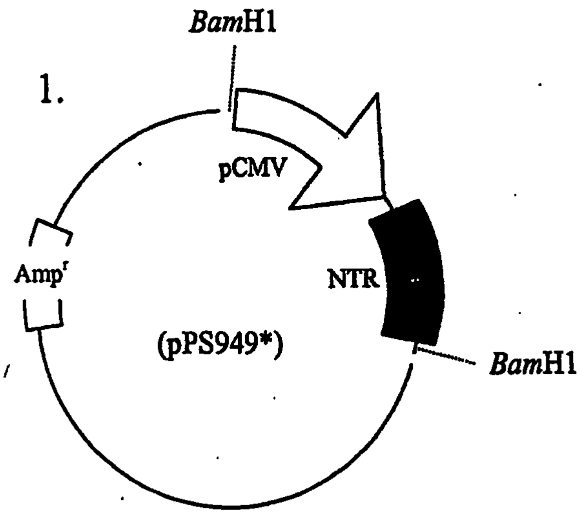
C

D

E

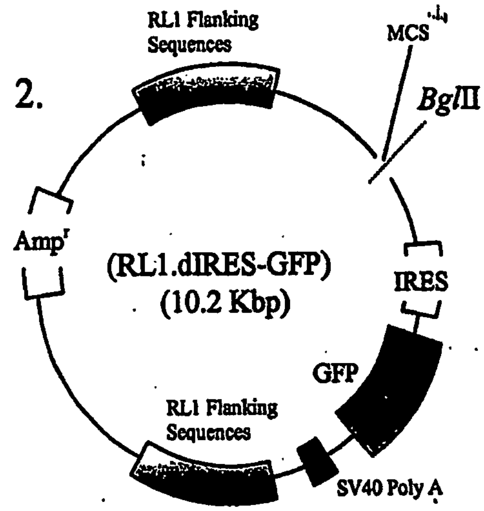
Figure 7

1.

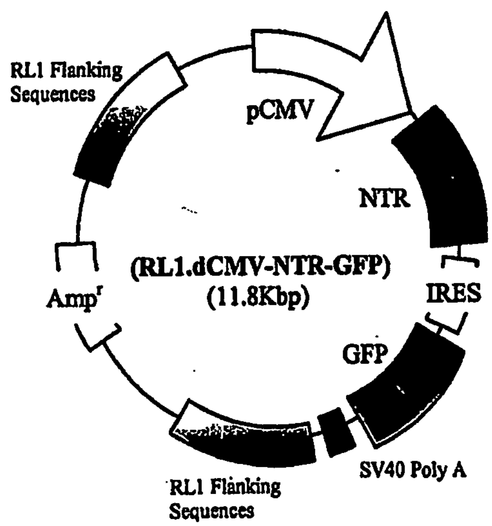


Digest with *Bam*HI and purify the pCMV-NTR fragment

2.



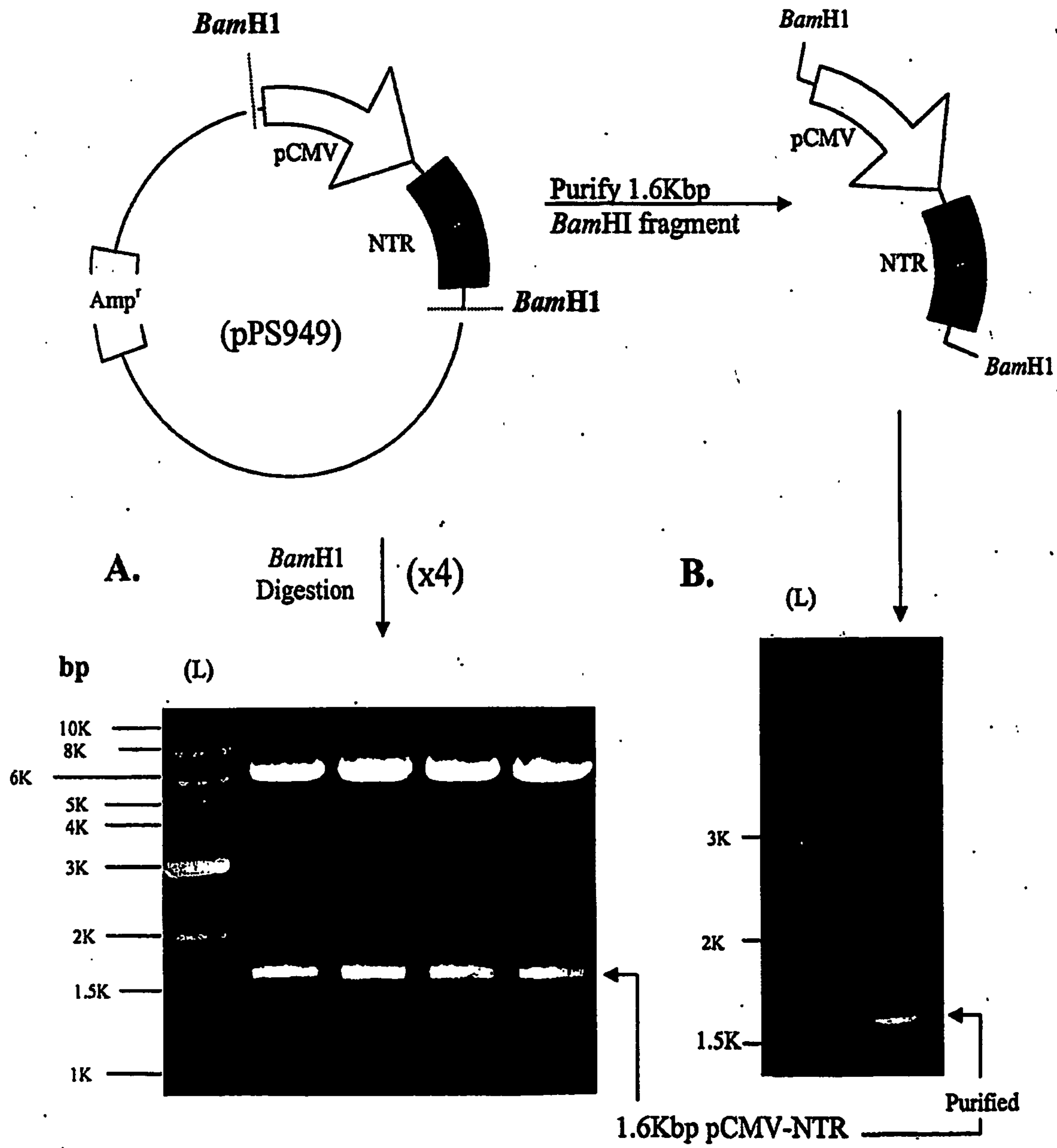
Digest with *Bgl*II and treat with CIP



3.

Clone pCMV-NTR (*Bam*HI ends) into *Bgl*II digested RL1.dIRES-GFP

Figure 8



BamH1
pCMV
NTR
Ampr
(pPS949)
BamH1
Purify 1.6Kbp
BamHI fragment
BamH1
pCMV
NTR
BamH1
A.
BamH1
Digestion
(x4)
B.
(L)
bp
(L)
10K
8K
6K
5K
4K
3K
2K
1.5K
1K
3K
2K
1.5K
Purified
1.6Kbp pCMV-NTR

Figure 9

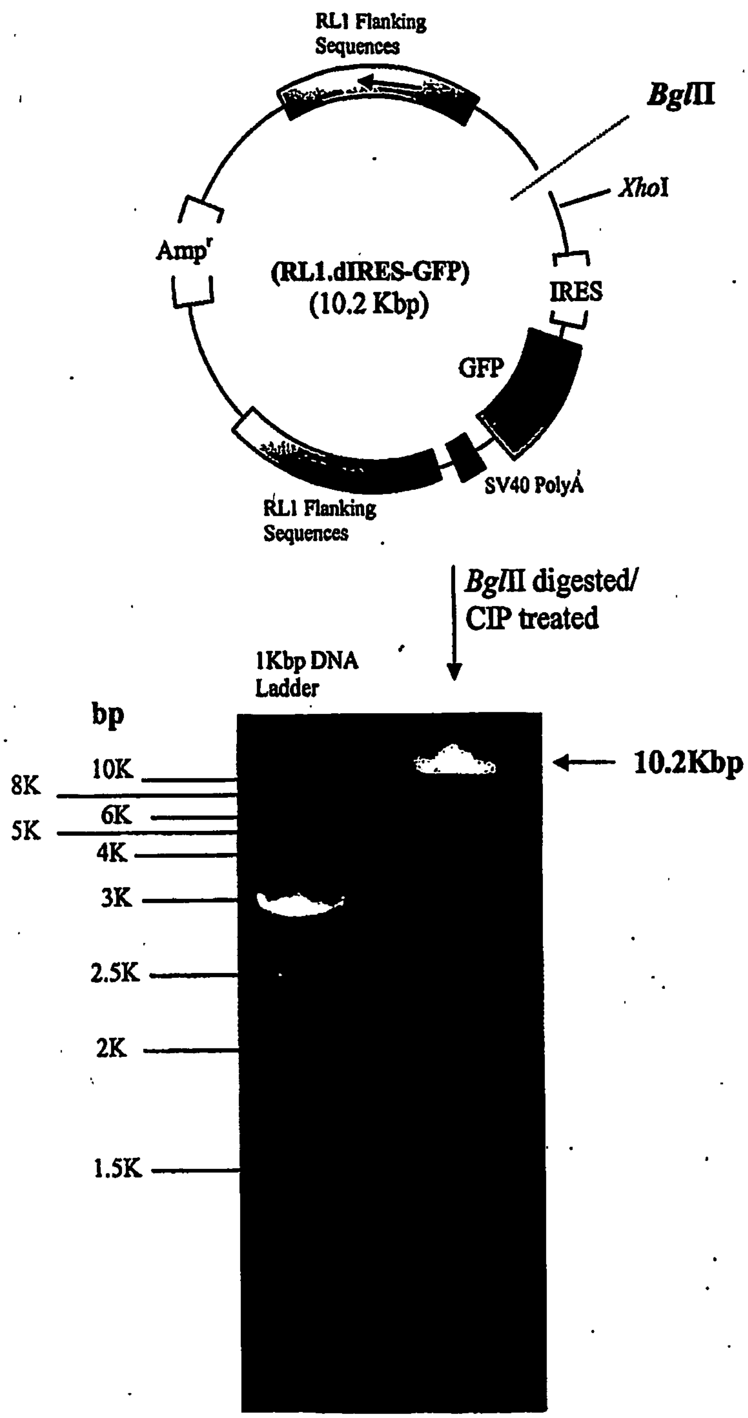
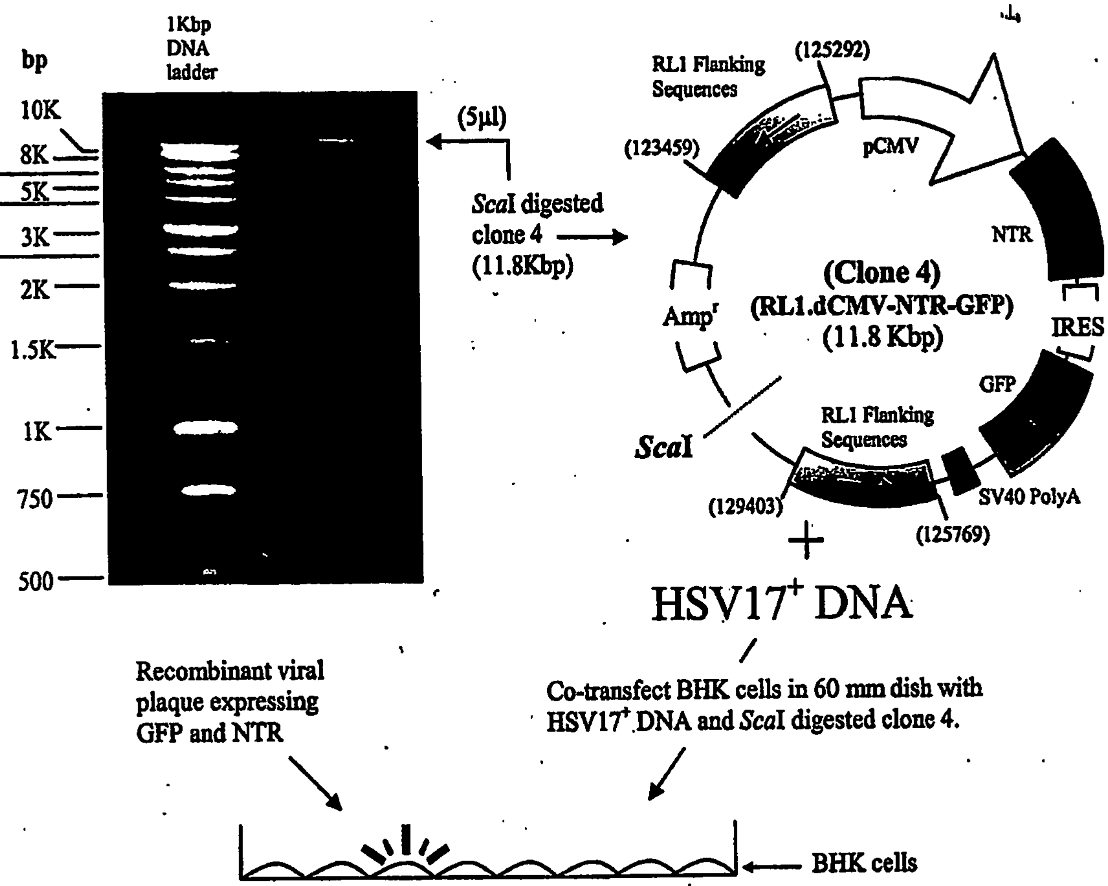

RL1 Flanking
Sequences
BglII
XhoI
Ampr
(RL1.dIRES-GFP)
(10.2 Kbp)
IRES
GFP
SV40 PolyA
RL1 Flanking
Sequences
BglII digested/
CIP treated
1Kbp DNA
Ladder
bp
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K
10.2Kbp

Figure 10

A.



B.

HSV1716/CMV-NTR/GFP Fraction	Titre
Cell Released (CR)	1.00×10^9 pfu/ml
Cell Associated (CA)	2.38×10^9 pfu/ml

Figure 12

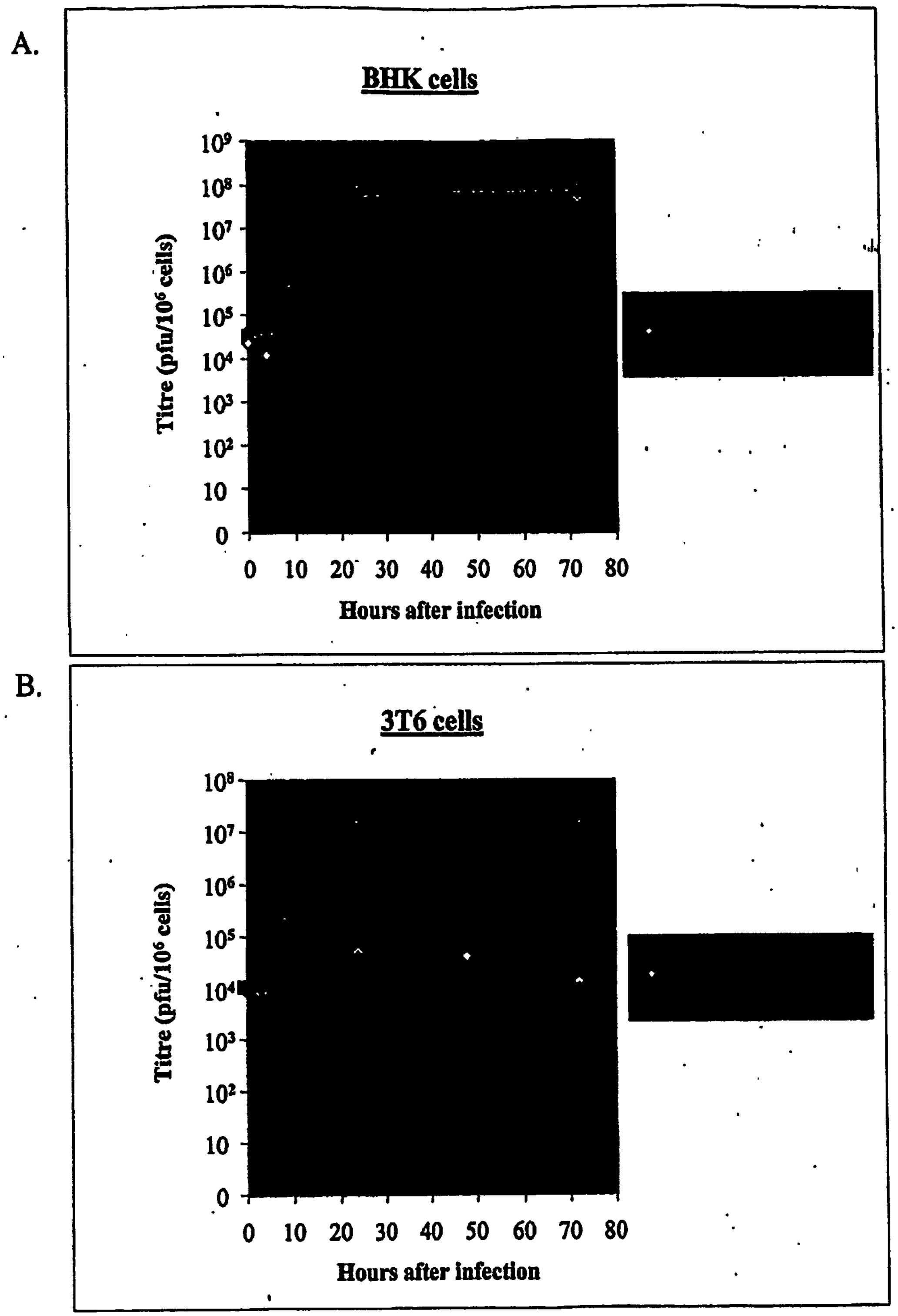

A.
BHK cells
Titre (pfu/10^6 cells)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
0 10 20 30 40 50 60 70 80
Hours after infection
B.
3T6 cells
Titre (pfu/10^6 cells)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
0 10 20 30 40 50 60 70 80
Hours after infection

Figure 13

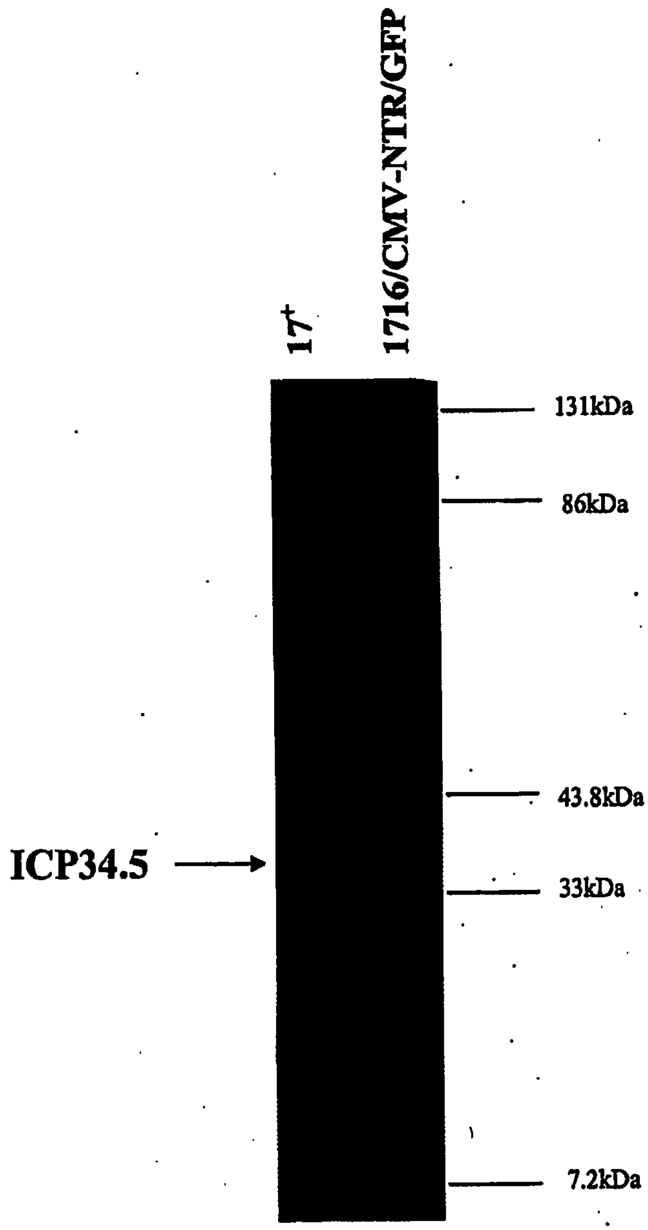


Figure 14

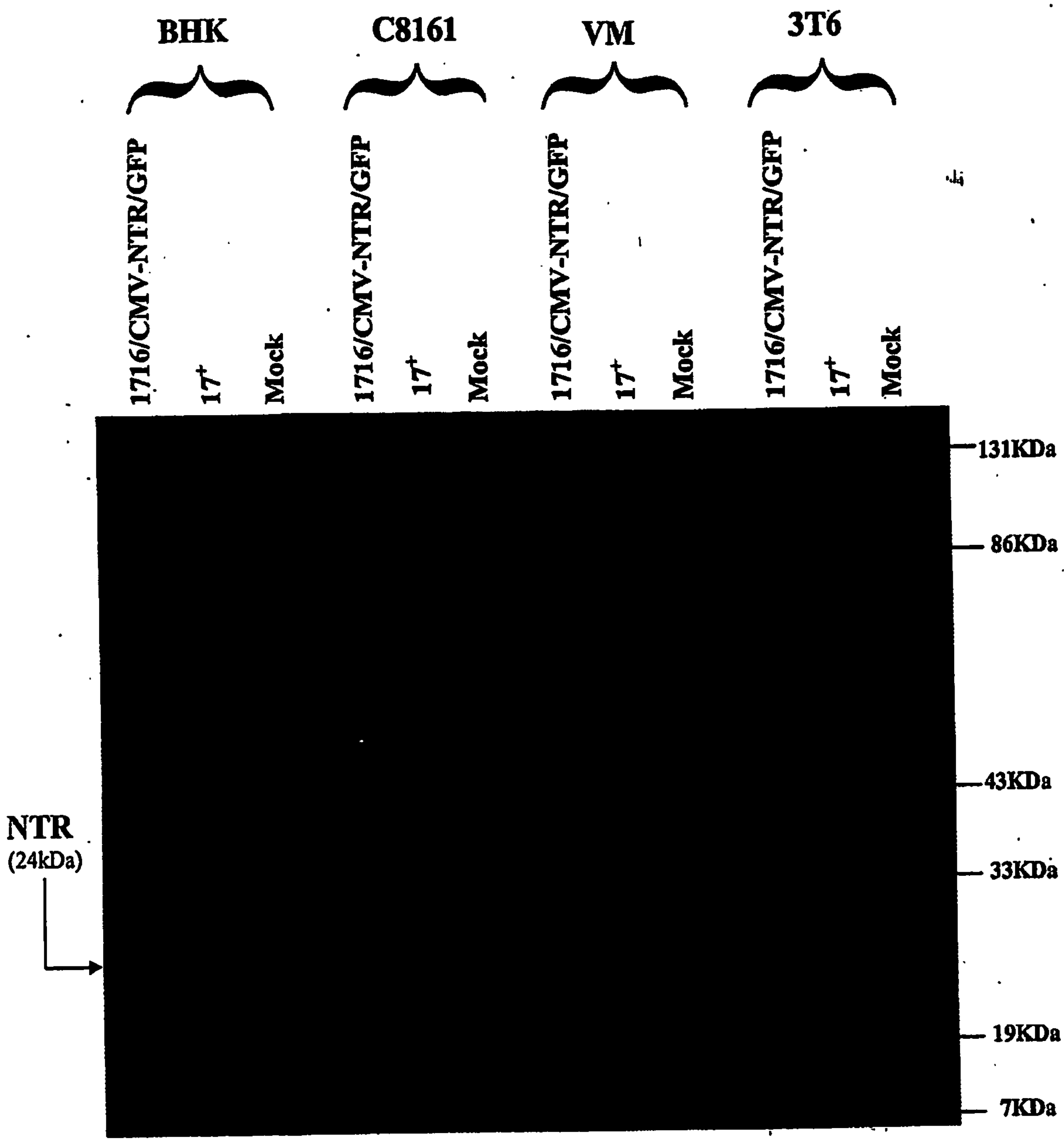

BHK
C8161
VM
3T6
1716/CMV-NTR/GFP
17+
Mock
1716/CMV-NTR/GFP
17+
Mock
1716/CMV-NTR/GFP
17+
Mock
1716/CMV-NTR/GFP
17+
Mock
131KDa
86KDa
43KDa
33KDa
19KDa
7KDa
NTR
(24kDa)

Figure 15

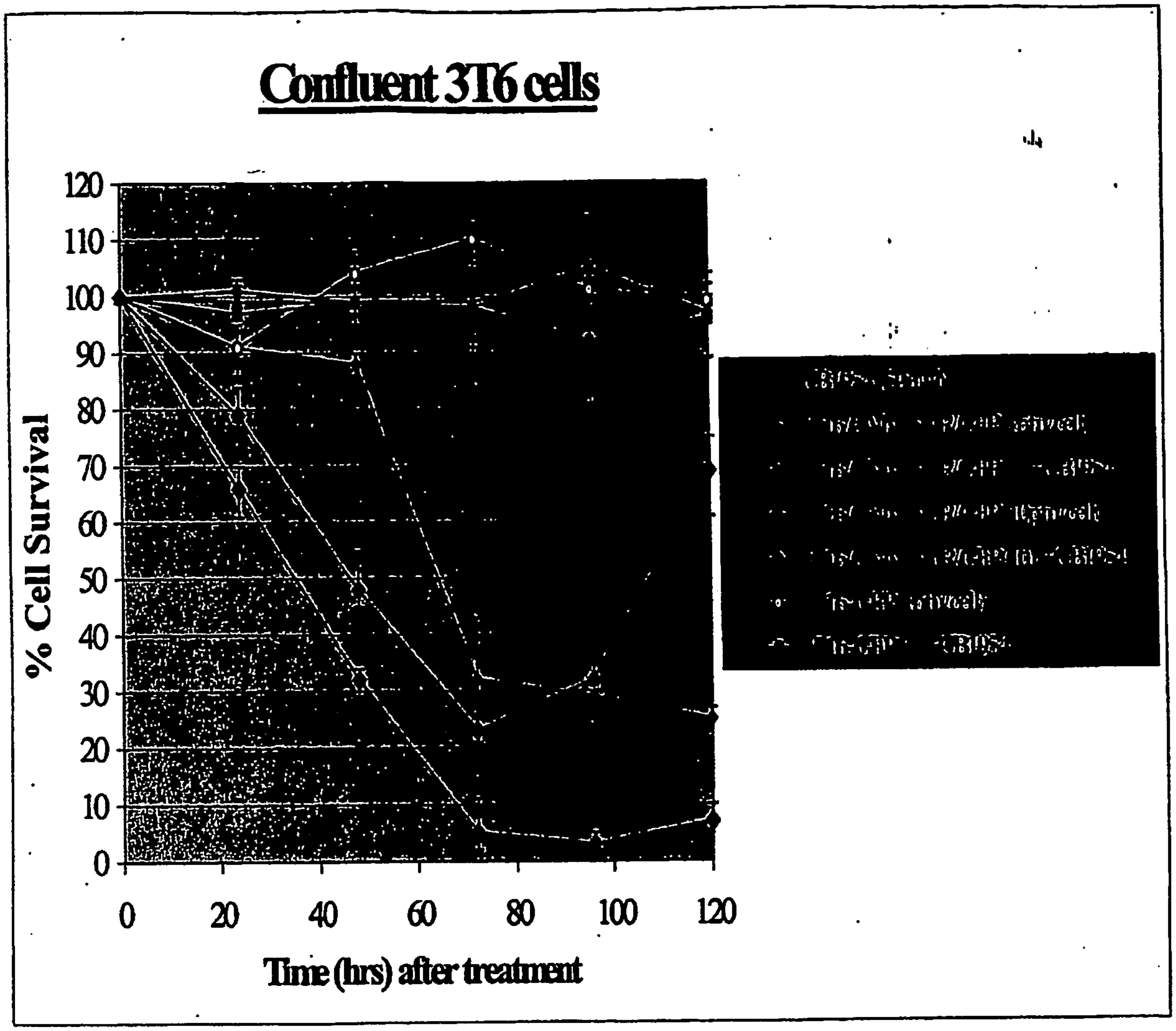

Confluent 3T6 cells
% Cell Survival
120
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (hrs) after treatment

Figure 16

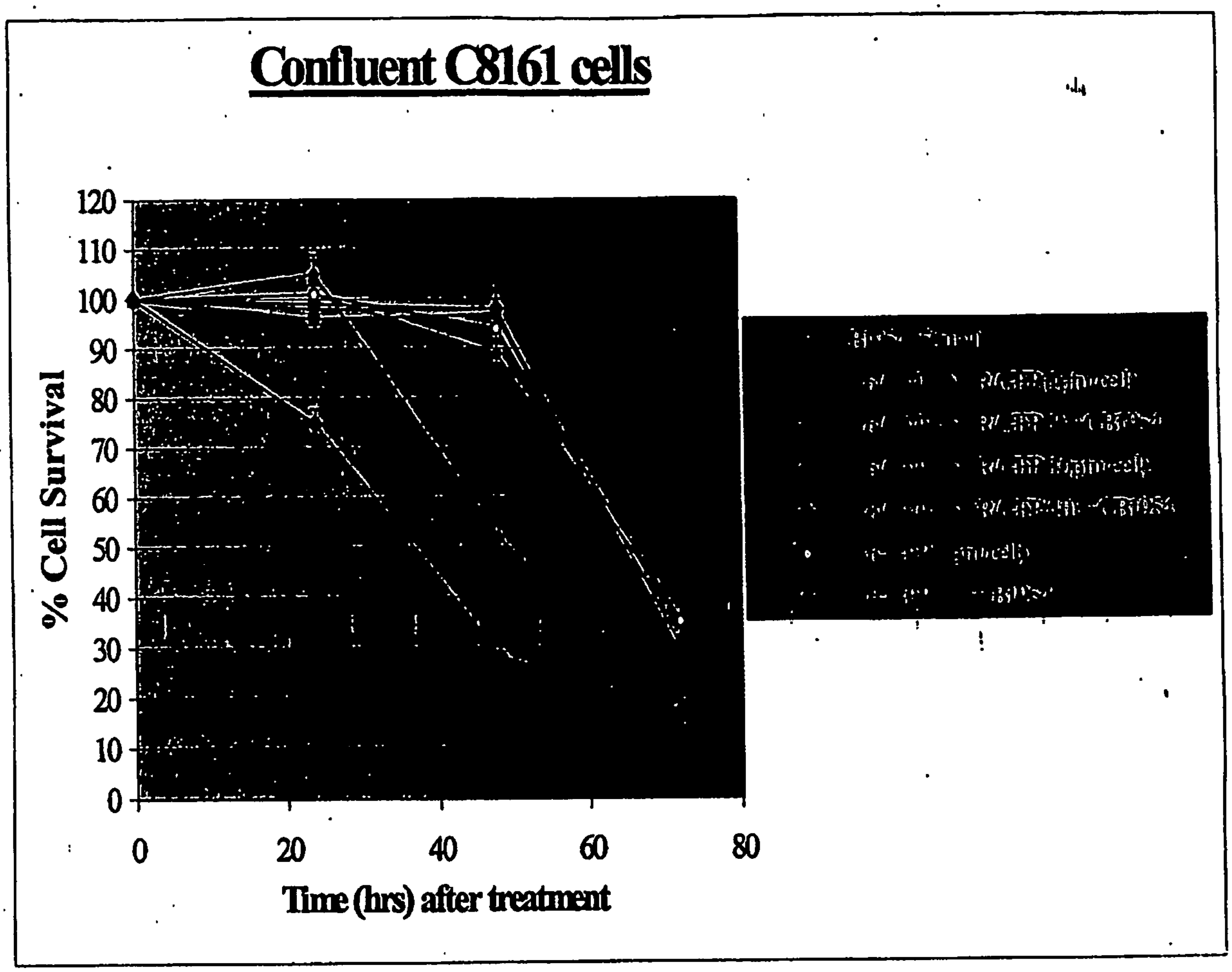

Confluent C8161 cells
120
110
100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0
20
40
60
80
Time (hrs) after treatment

Figure 17

A.

B.

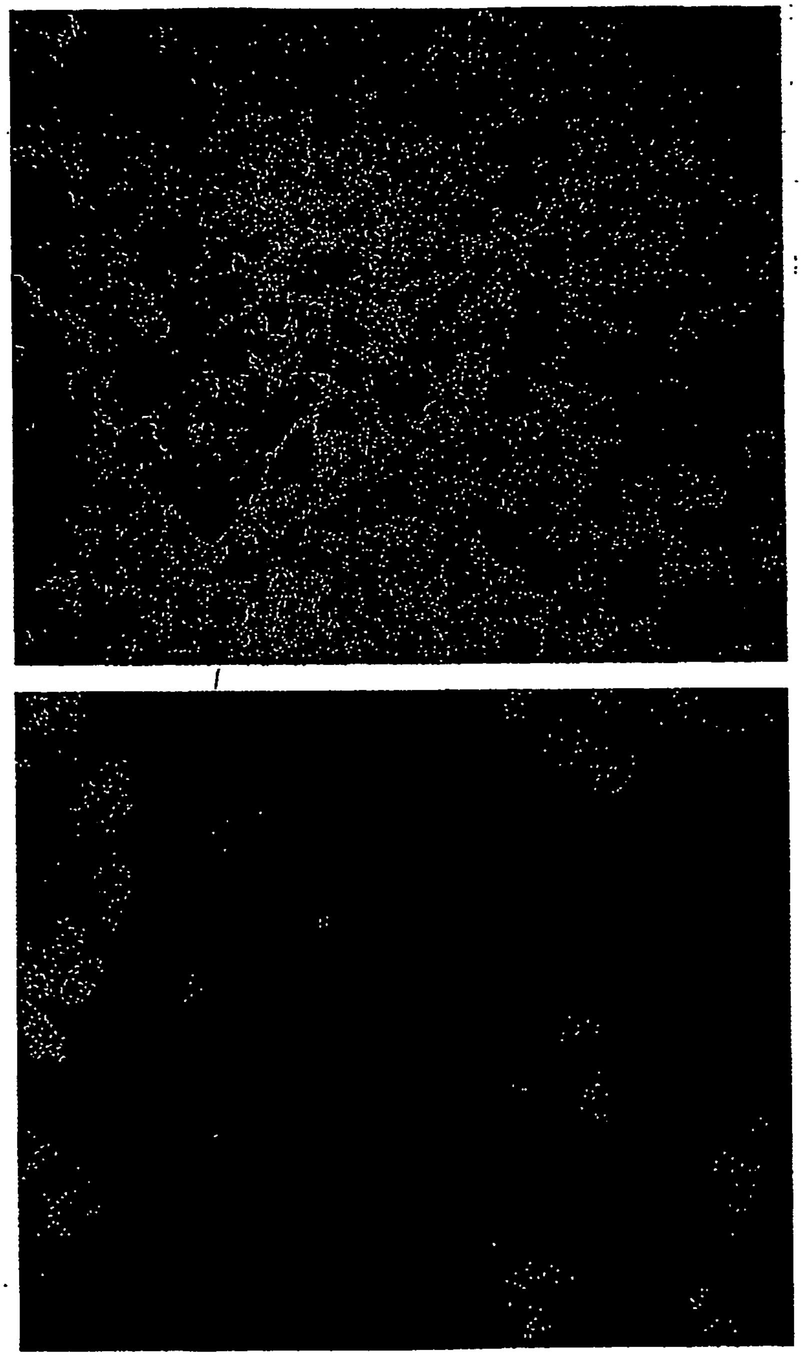
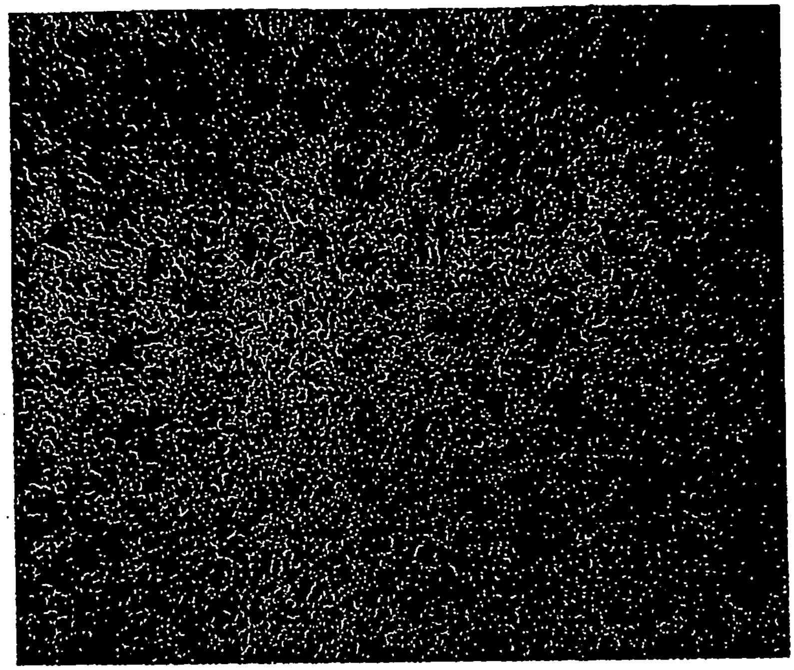


Figure 18

A.



B.

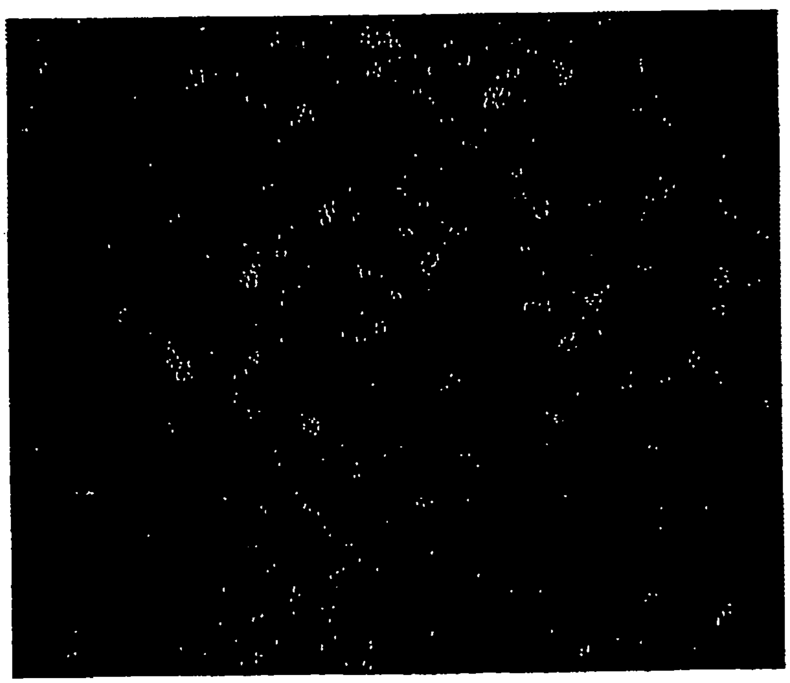


Figure 19

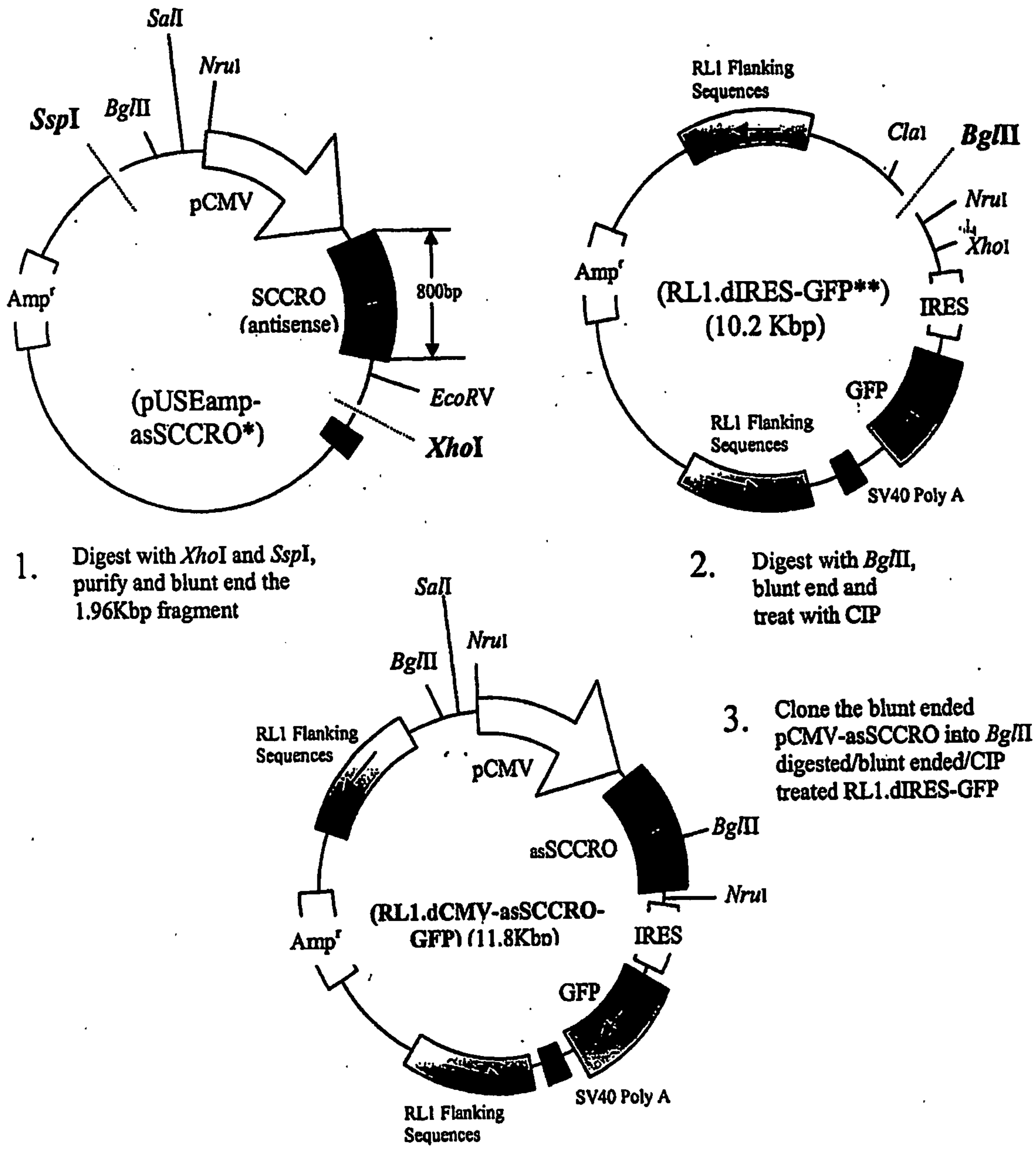
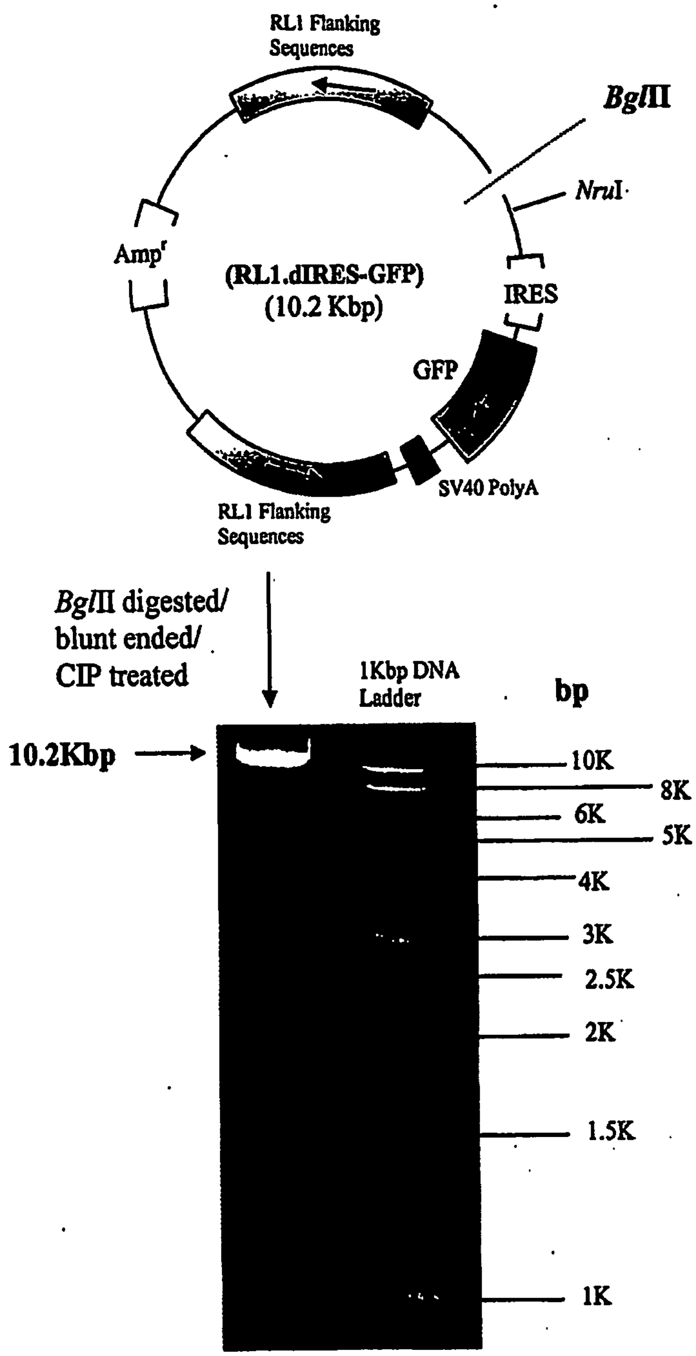
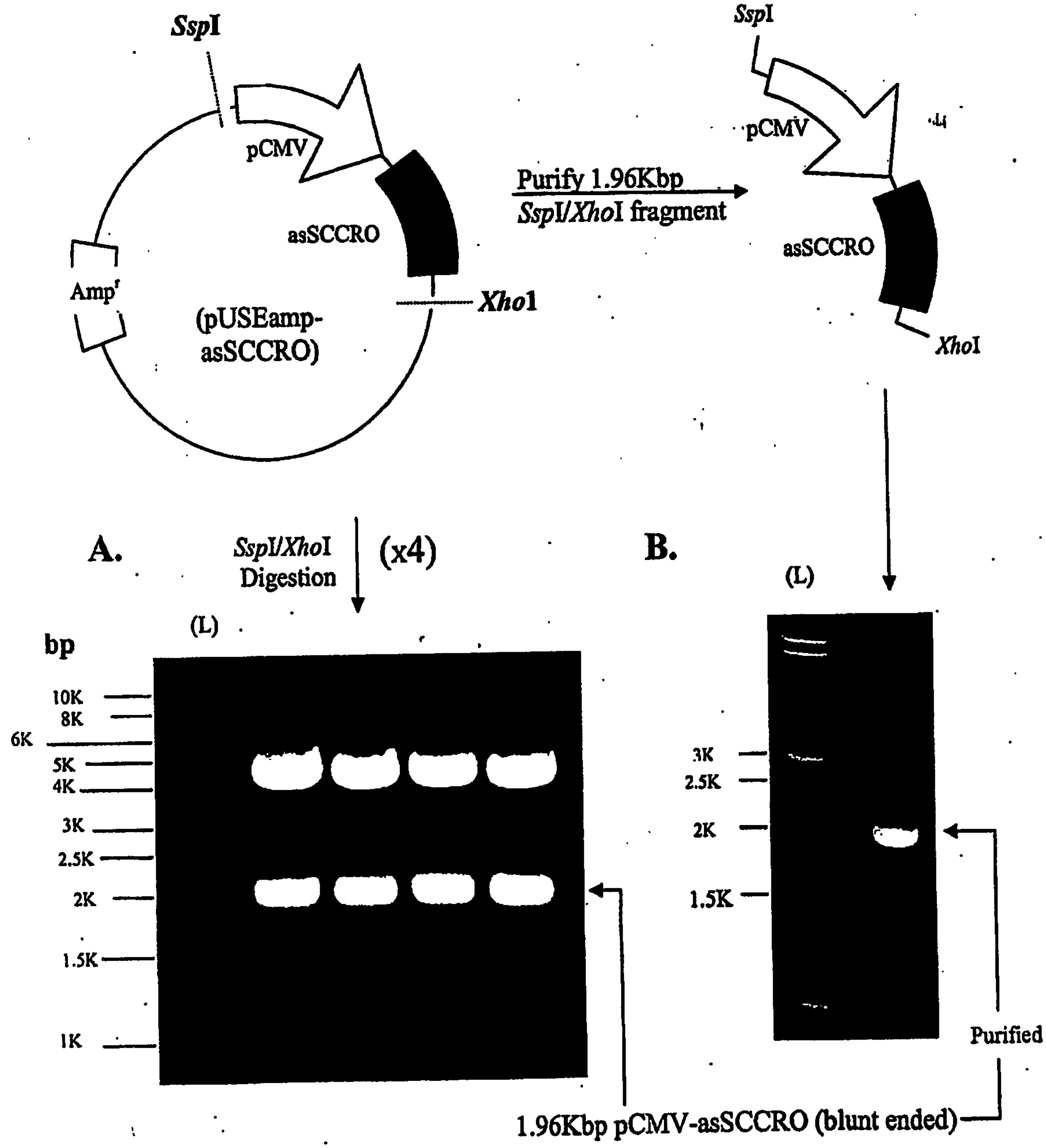

SalI
NruI
SspI
BglII
pCMV
Amp^r
SCCRO
(antisense)
800bp
(pUSEamp-
asSCCRO*)
EcoRV
XhoI
RL1 Flanking
Sequences
ClaI
BglII
NruI
XhoI
Amp^r
(RL1.dIRES-GFP**)
(10.2 Kbp)
IRES
GFP
RL1 Flanking
Sequences
SV40 Poly A
1. Digest with XhoI and SspI, purify and blunt end the 1.96Kbp fragment
2. Digest with BglII, blunt end and treat with CIP
SalI
BglII
NruI
RL1 Flanking
Sequences
pCMV
BglII
asSCCRO
NruI
Amp^r
(RL1.dCMV-asSCCRO-
GFP) (11.8Kbp)
IRES
GFP
SV40 Poly A
RL1 Flanking
Sequences
3. Clone the blunt ended pCMV-asSCCRO into BglII digested/blunt ended/CIP treated RL1.dIRES-GFP

Figure 20



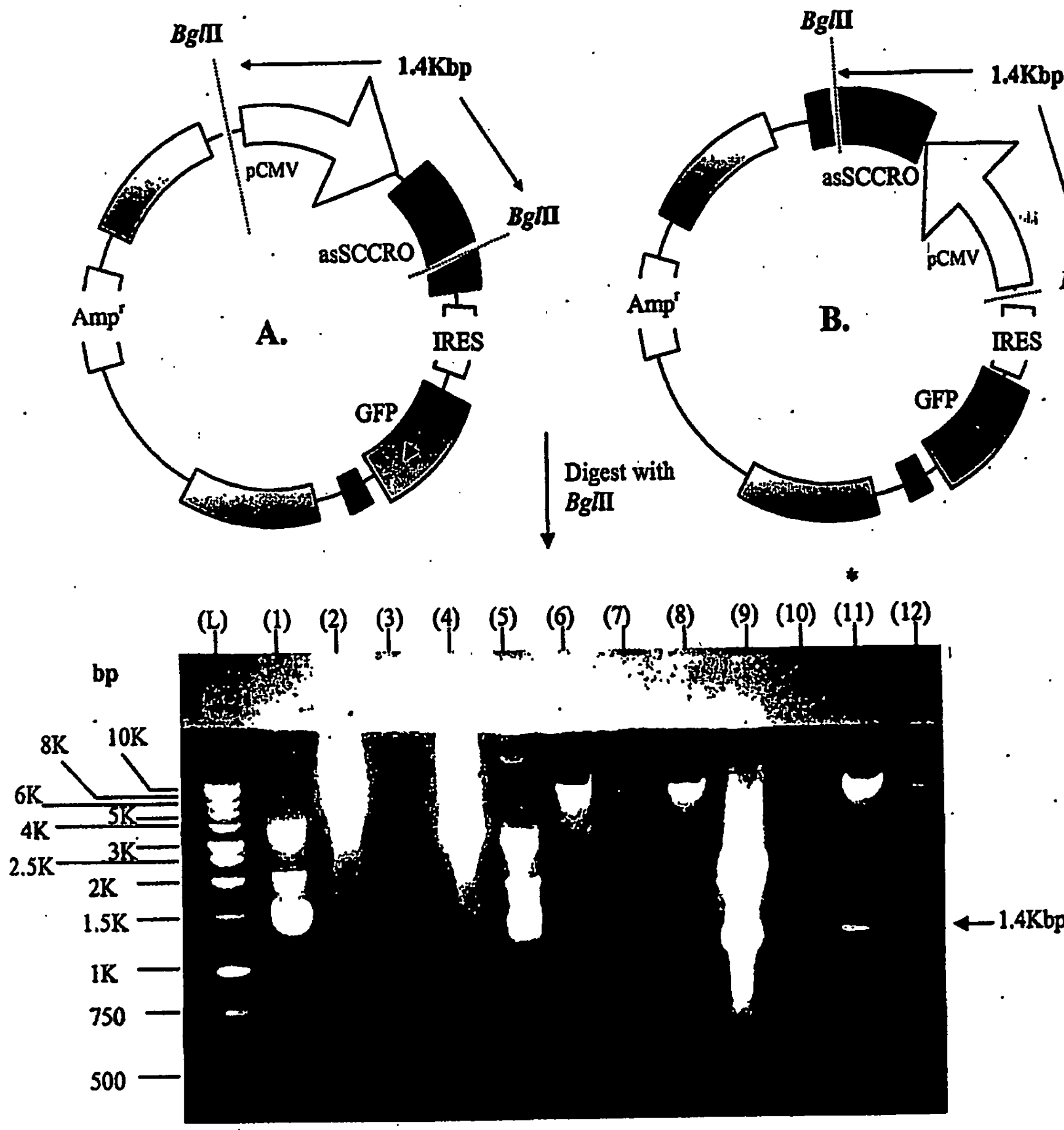
RL1 Flanking Sequences
BglII
NruI
Ampr
(RL1.dIRES-GFP)
(10.2 Kbp)
IRES
GFP
SV40 PolyA
RL1 Flanking Sequences
BglII digested/
blunt ended/
CIP treated
1Kbp DNA Ladder
bp
10.2Kbp
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K
1K

Figure 21



SspI
pCMV
asSCCRO
Ampr
(pUSEamp-
asSCCRO)
XhoI
Purify 1.96Kbp
SspI/XhoI fragment
SspI
pCMV
asSCCRO
XhoI
A.
SspI/XhoI
Digestion
(x4)
bp
(L)
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K
1K
B.
(L)
3K
2.5K
2K
1.5K
Purified
1.96Kbp pCMV-asSCCRO (blunt ended)

Figure 22



BglII
1.4Kbp
pCMV
asSCCRO
BglII
Ampr
A.
IRES
GFP
BglII
1.4Kbp
asSCCRO
pCMV
BglII
Ampr
B.
IRES
GFP
Digest with BglII
(L) (1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11) (12)
bp
8K
10K
6K
5K
4K
3K
2.5K
2K
1.5K
1K
750
500
1.4Kbp

Figure 23

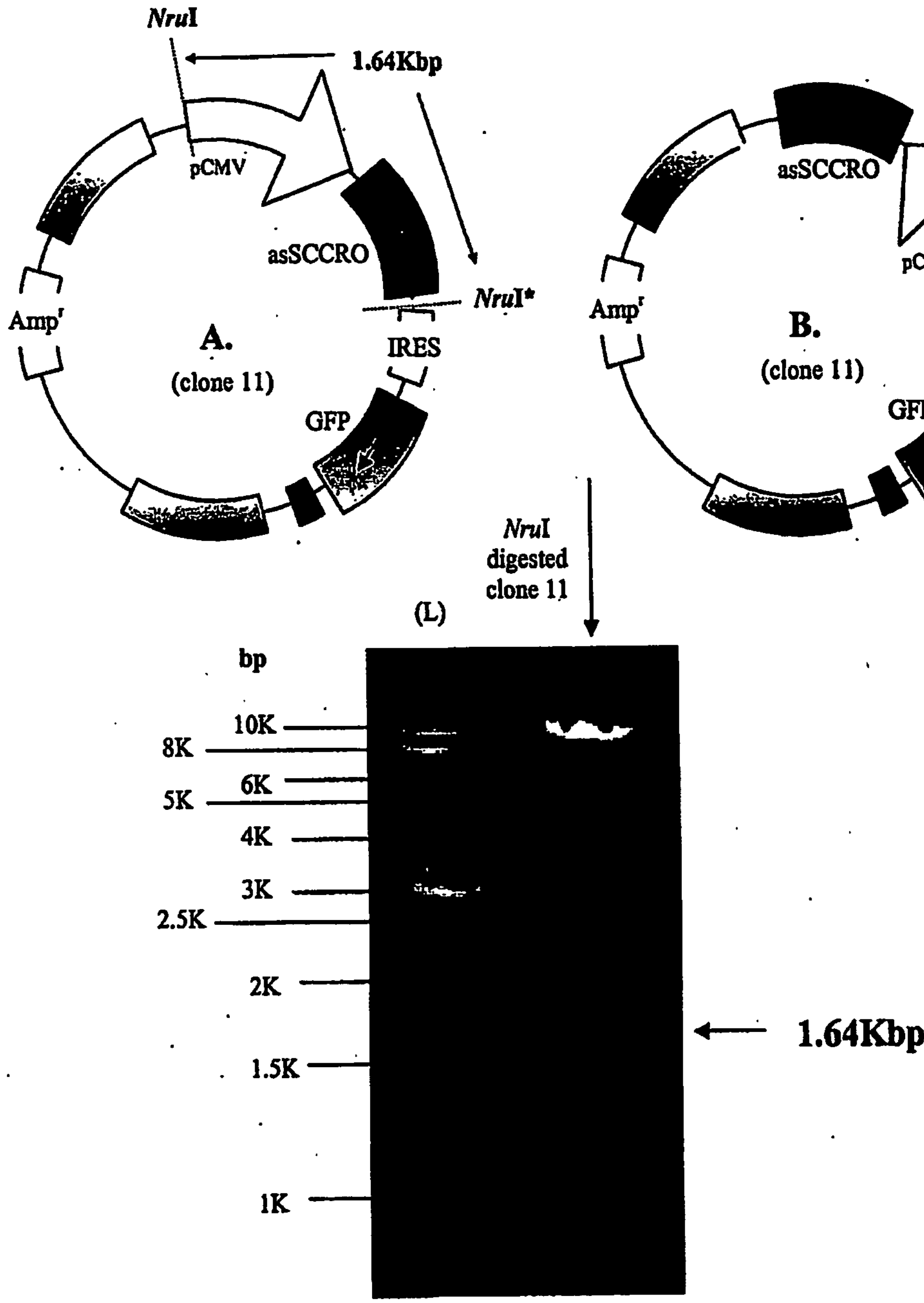
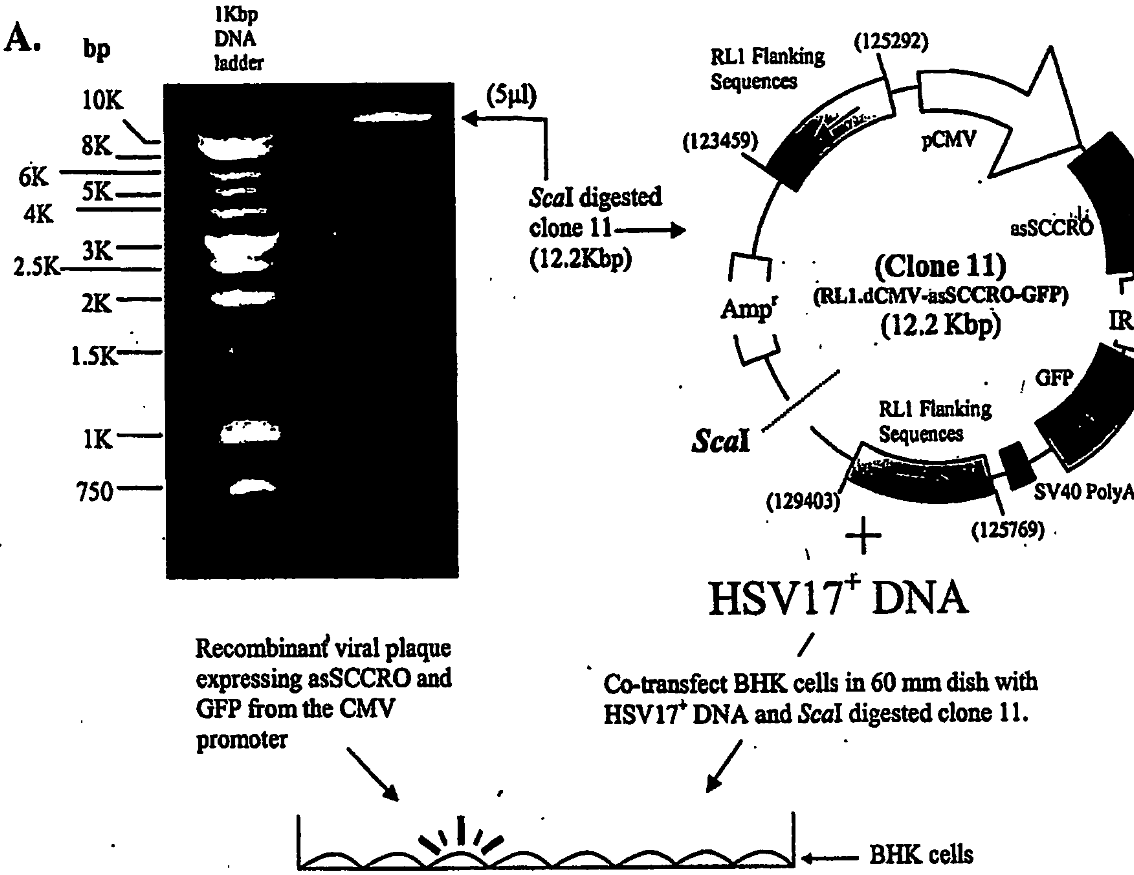

NruI
1.64Kbp
pCMV
asSCCRO
NruI*
Ampr
A.
(clone 11)
IRES
GFP
asSCCRO
pCMV
NruI
NruI*
Ampr
B.
(clone 11)
IRES
GFP
NruI
digested
clone 11
(L)
bp
10K
8K
6K
5K
4K
3K
2.5K
2K
1.5K
1K
1.64Kbp

Figure 24

A.



B.

HSV1716/CMV-asSCCRO/GFP Fraction	Titre
Combined	1.2×10^{10} pfu/ml

Figure 25

SCC15

584

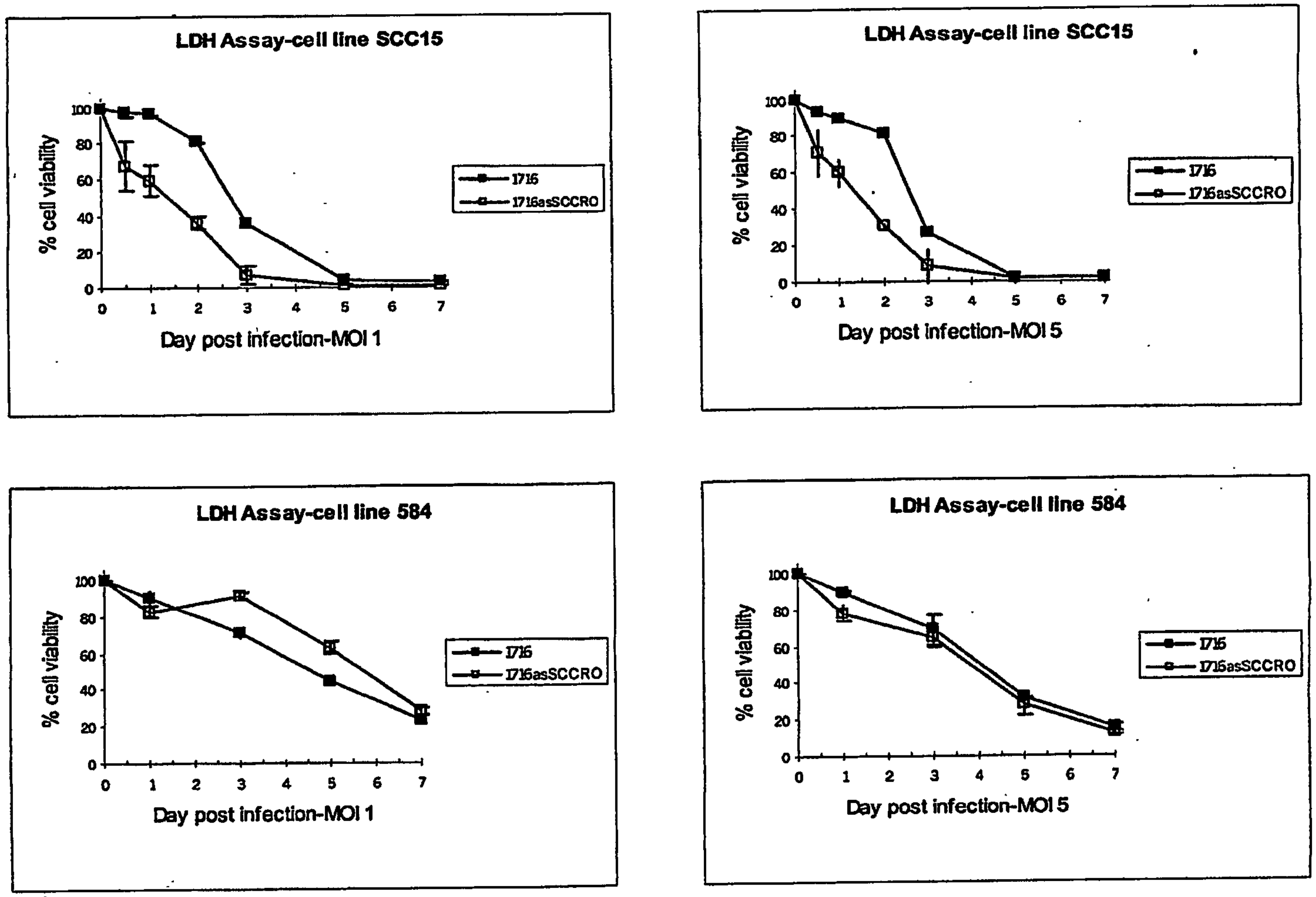


Figure 26

1483

1986

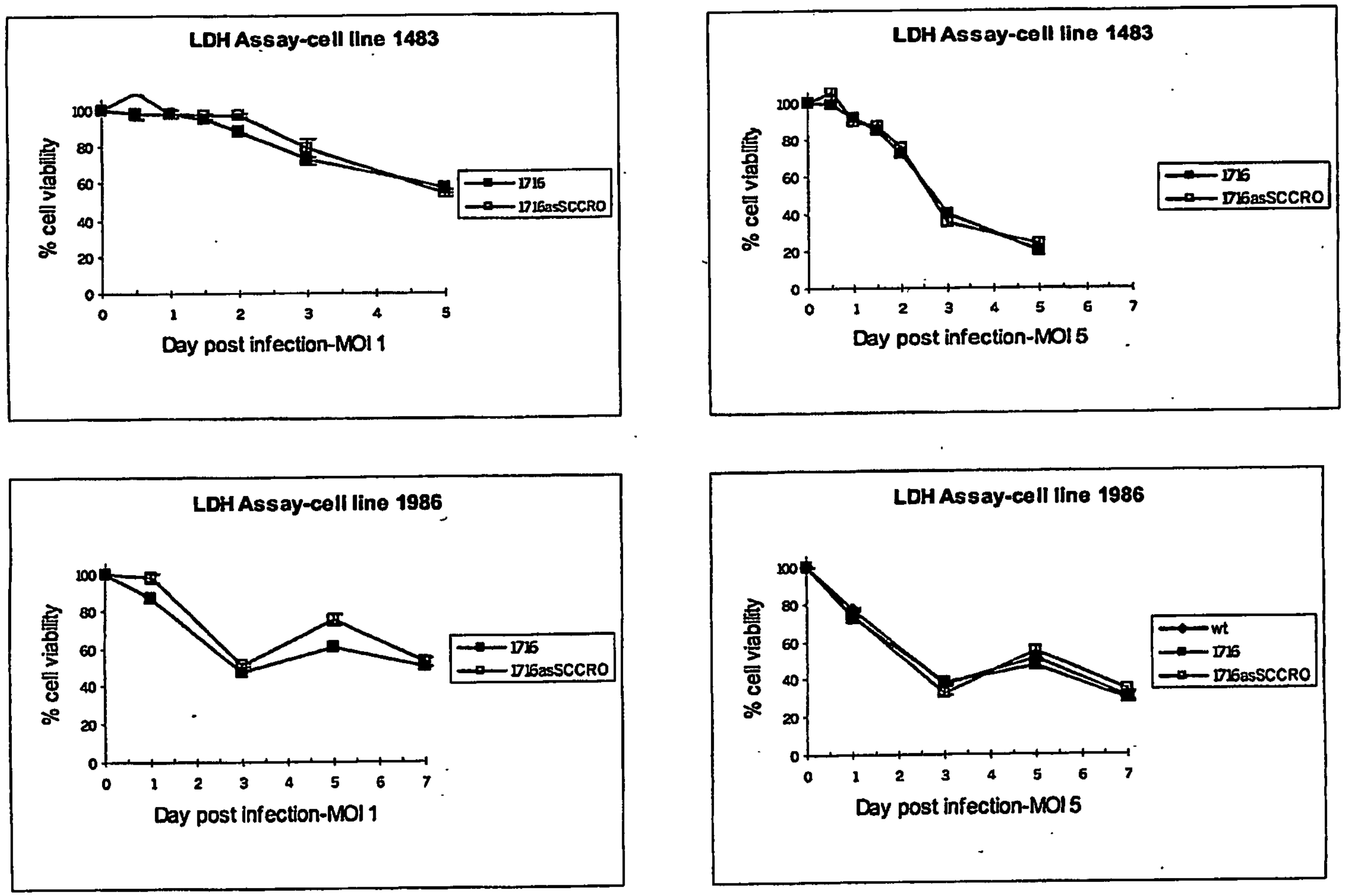
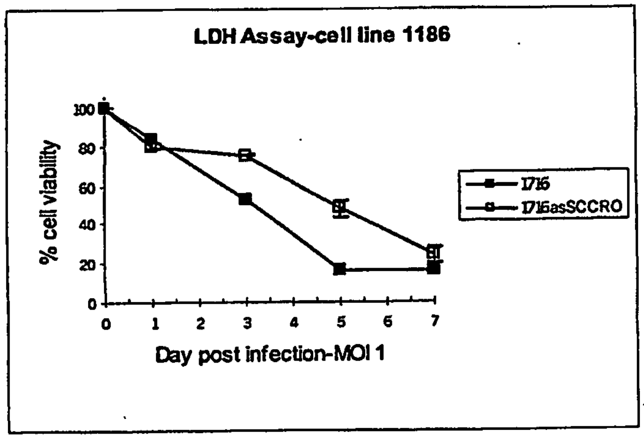
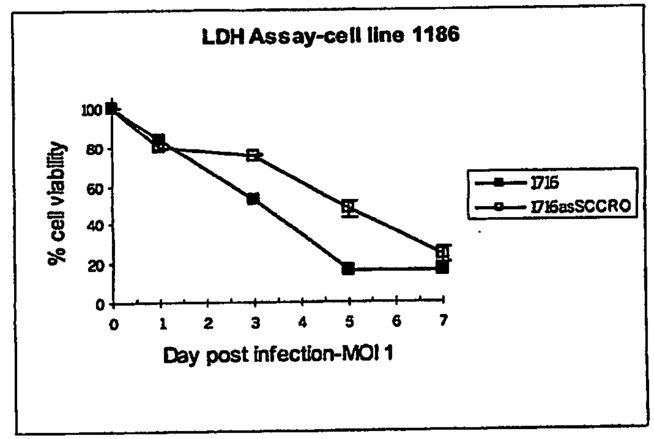


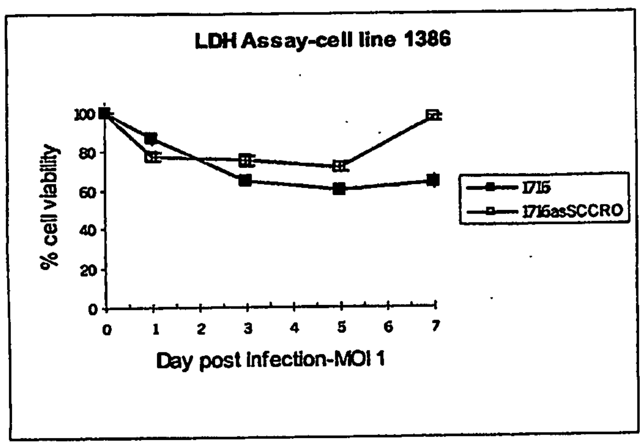
Figure 27

1186





1386



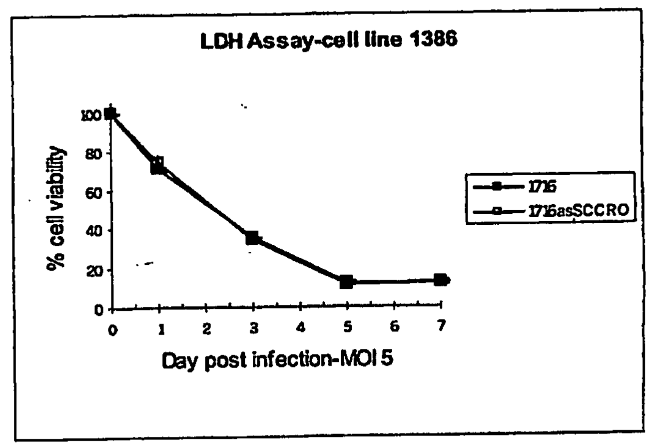


Figure 28

SCC15

584

1483

1986

1186

1386

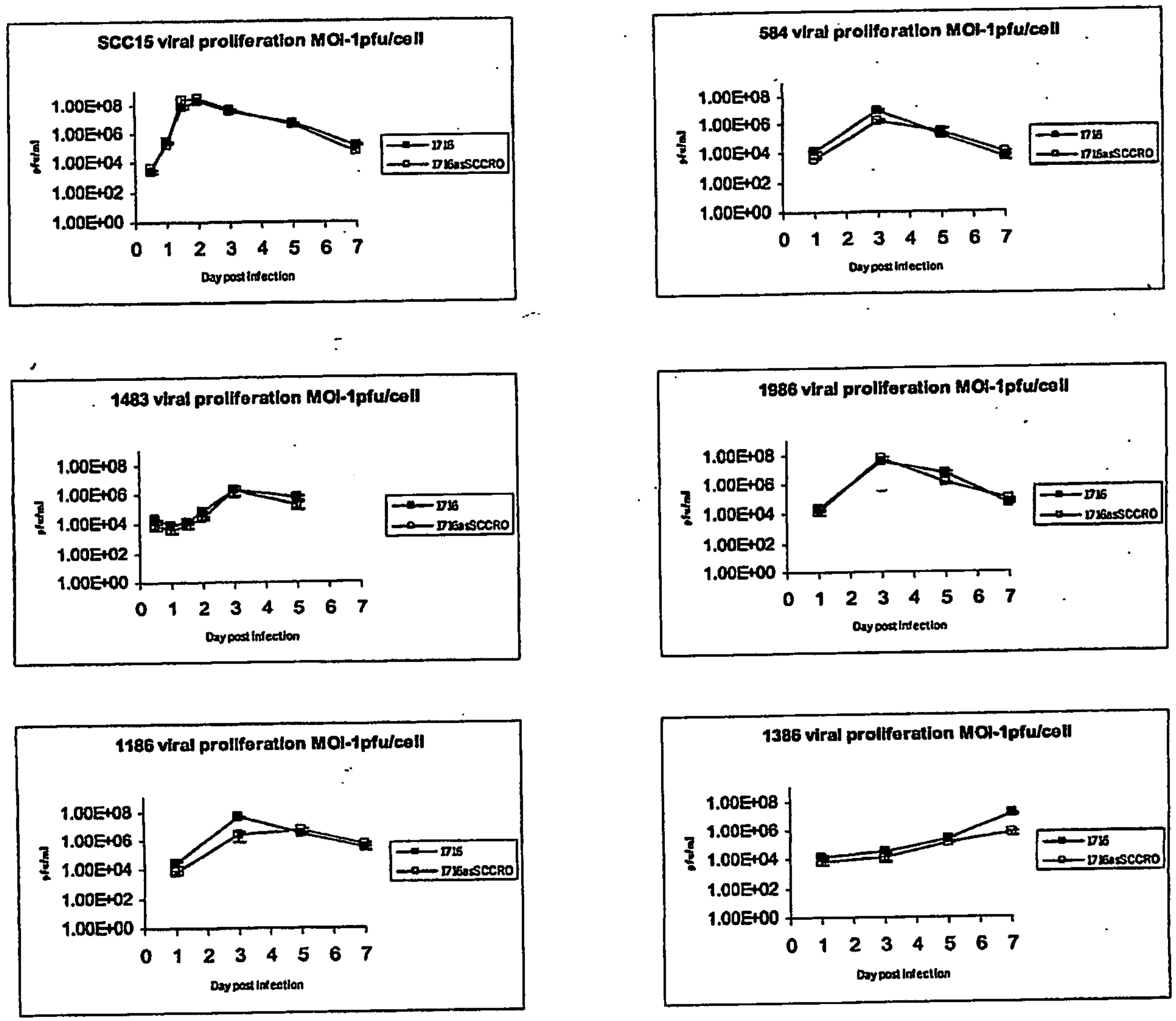


Figure 29

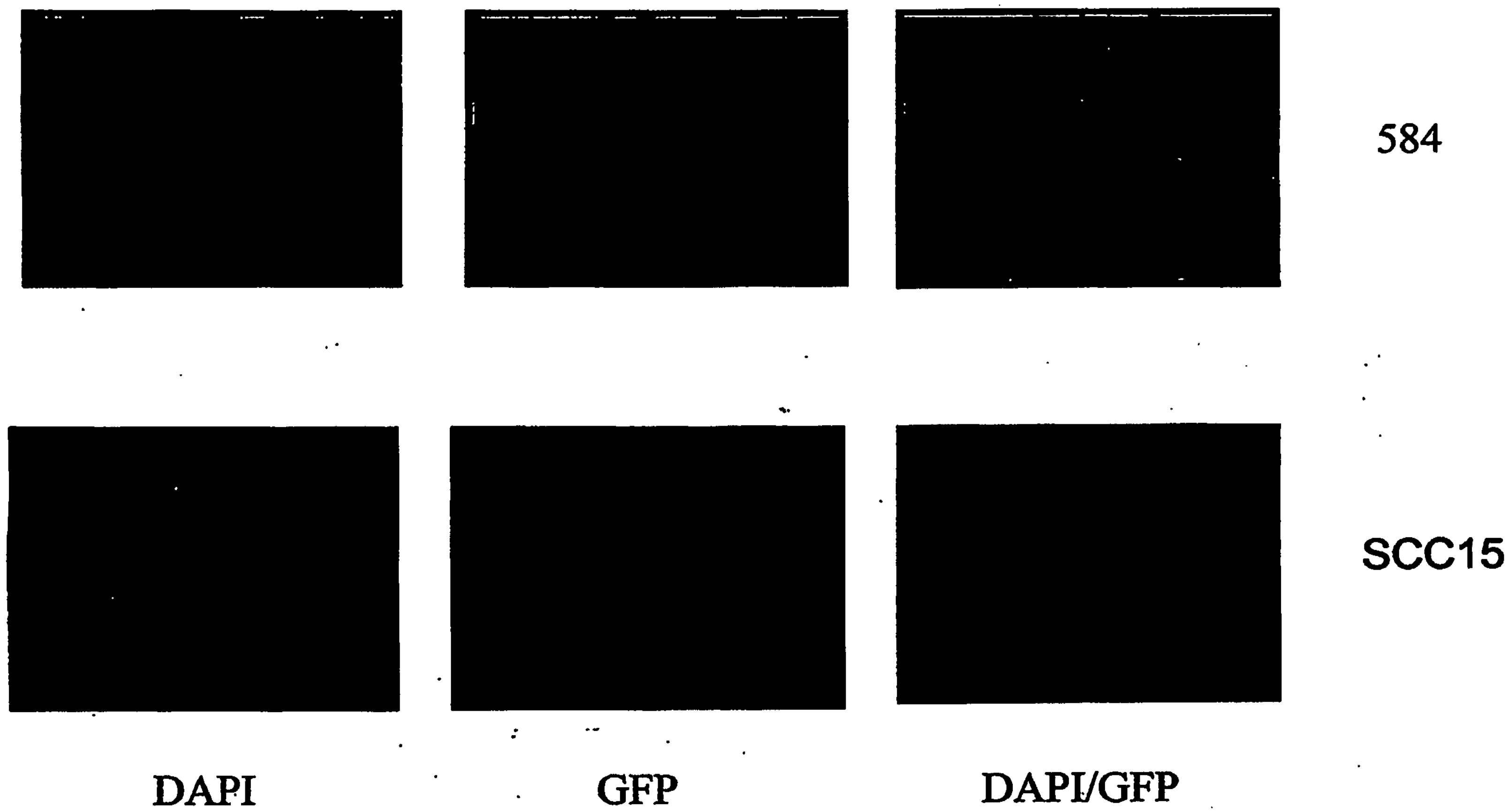


Figure 30

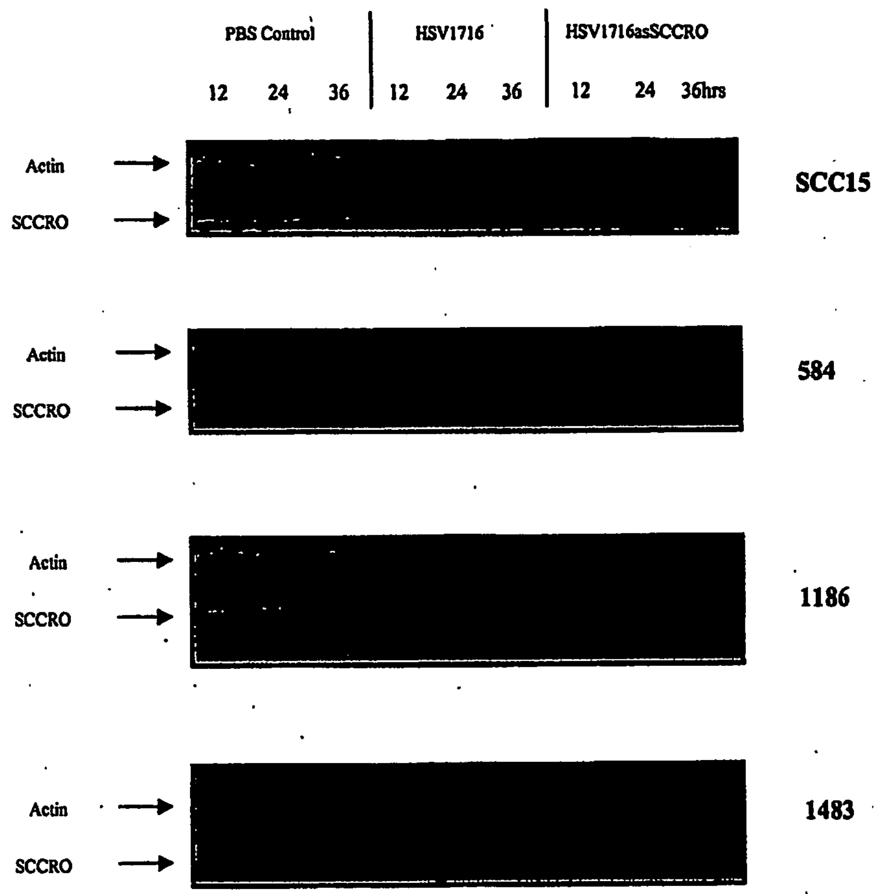
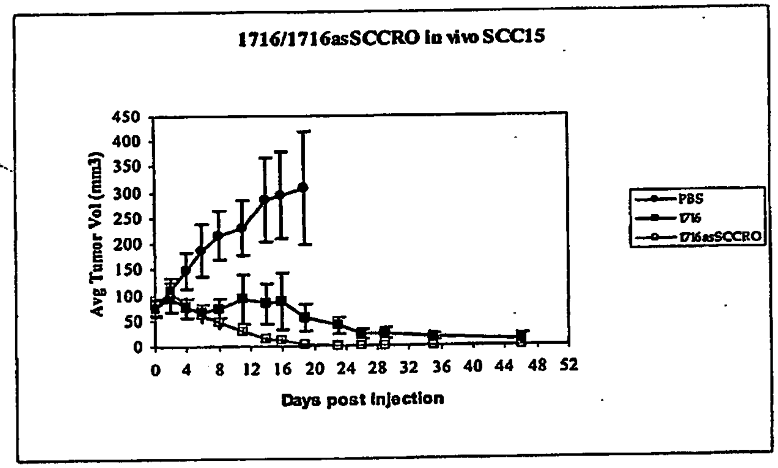
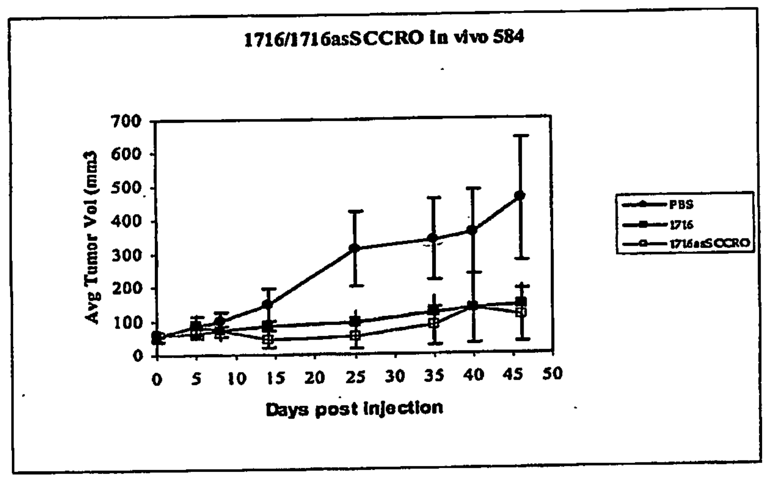

PBS Control
HSV1716
HSV1716asSCCRO
12
24
36
12
24
36
12
24
36hrs
Actin
SCCRO
SCC15
Actin
SCCRO
584
Actin
SCCRO
1186
Actin
SCCRO
1483

Figure 31

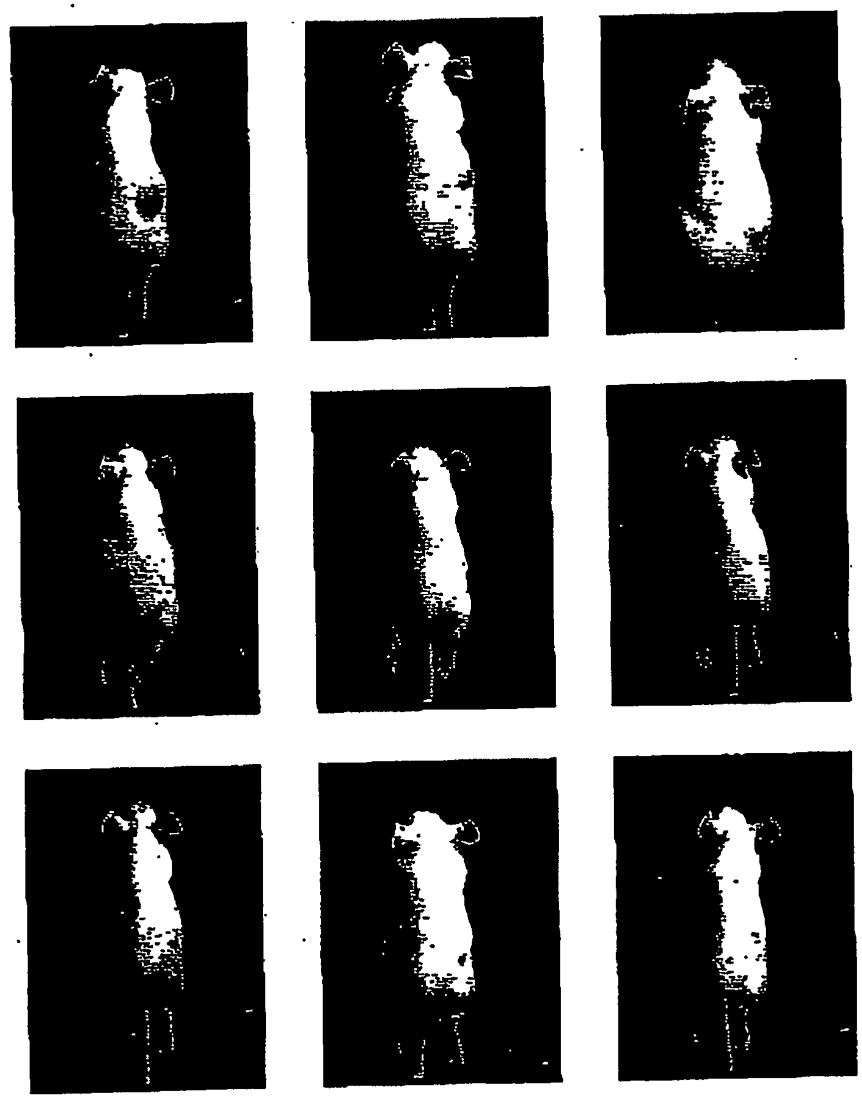


SCC15



584

Figure 32



PBS

1716as
SCCRO

1716

Figure 33